UNIVERSITY OF ALBERTA

APOPTOSIS INHIBITION TO PROMOTE β-CELL SURVIVAL IN ISLET TRANSPLANTATION

BY

©

JULIET ANN EMAMAULLEE

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DEDICATION

This thesis is dedicated to the patients in the Edmonton Clinical Islet Transplantation Program. Their commitment to and personal investment in improving the lives of patients with diabetes has been a constant inspiration for me.

ABSTRACT

The Edmonton Protocol for islet transplantation typically requires ≥2 islet donors to achieve euglycemia in a single recipient, primarily because soon after portal infusion the majority of the transplanted cells undergo apoptosis due to hypoxia and hypoxia-reperfusion injury. Several strategies have been explored to prevent β-cell death post-transplant, but no anti-apoptotic gene manipulation has thus far demonstrated a significant protective effect in transplanted islets.

This thesis focuses on the hypothesis that survival of β-cells would be enhanced if a potent, naturally occurring inhibitor of apoptosis protein called XIAP was overexpressed in islet grafts. First, a growth-regulatable murine β-cell line was used as a model for β-cells within islets, and XIAP was delivered using an adenoviral vector. XIAP overexpressing β-cells were markedly resistant to apoptosis in an ischemia-reperfusion injury model system and following exposure to cytokines in vitro. When Ad-XIAP transduced β-cells were transplanted into immunodeficient mice, the grafts were able to reverse diabetes quite rapidly compared to control cells. Human islets were also transduced with Ad-XIAP, and this prevented β-cell apoptosis following hypoxia and reoxygenation. Furthermore XIAP overexpression dramatically reduced the number of human islets required to reverse diabetes in immunodeficient mice. These results suggest that by overexpressing XIAP in the immediate post-transplant period, human islets from a single donor might be used to effectively treat two diabetic recipients.

Even with the success of the Edmonton protocol, an alternate source of islet tissue must be developed if β-cell replacement therapy is to see widespread

clinical application. Neonatal porcine islets represent one potential source of tissue, with advantages of economy, reproducibility of isolation, excellent survival in vitro, and proven function in vivo in experimental animals. We found that this tissue has yet another advantage—a remarkable capacity to resist apoptosis and maintain glucose-responsive insulin secretion when exposed to severe stresses such as hypoxia and re-oxygenation. This tissue was found to express continuous high levels of XIAP, whereas adult islets did not.

These studies suggest that β-cell apoptosis post-transplant can be dramatically reduced by XIAP overexpression. This should reduce the shedding of donor antigen and thus diminish immune stimulation in the recipient.

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LIST OF ABBREVIATIONS

APC	Antigen Presenting Cell
βGAL	βGalactosidase
BH3	BCL-2 Homology 3
BIR	Baculoviral Inhibitor of Apoptosis Repeat
BSA	Bovine Serum Albumin
CAR	Coxsackievirus and Adenovirus Receptor
CARD	Caspase Recruitment Domain
cFLIP	Cellular FLICE-Inhibitory Protein
DAPI	4',6-diamino-2-phenylindol
DCCT	Diabetes Control and Complications Trial
DISC	Death Inducibing Signaling Complex
DMEM	Dulbecco's Modified Eagle's Medium
FADD	Fas-Associated Death Domain
FITC	Fluorescein
GAD	Glutamic Acid Decarboxylase
GpX	Glutathione Peroxidase
hIAP	Human Inhibitor of Apoptosis Protein
HdAdV	Helper-Dependent Adenovirus
IAP	Inhibitor of Apoptosis Protein
IBMIR	Instant Blood-Mediated Inflammatory Reaction
IBMX	Isobutylmethohexane
IE	Islet Equivalent
IFNγ	Interferon-γ
IkK	IκB Kinase
IL-1β	Interleukin-1β
iNOS	Inducible Nitric Oxide Synthase
IRES	Internal Ribosome Entry Site
LPS	Lipopolysaccharide
MMF	Mycophenolate Mofetil
NAIP	Neuronal Apoptosis Inhibitory Protein
NF-κB	Nuclear Factor-κB
NO	Nitric Oxide
NOD	Non-Obese Diabetic
NPI	Neonatal Porcine Islet
PBMC	Peripheral Blood Mononuclear Cell
PE	Phycoerythrin
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
STZ	Streptozotocin
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TNF-α	Tumor Necrosis Factor-α
TRAF2	TNF Associated Receptor
TUNEL	Terminal dUTP Nick End Labeling

VEGF	Vascular Endothelial Growth Factor
VP	Virus Particle
XIAP	X-linked Inhibitor of Apoptosis Protein
zVAD-FMK	N-benzyloxycabonyl-Val-Ala-Asp-fluoromethylketone

Chapter 1

Loss of β-cell Mass in Islet Transplantation

1.1 Therapeutic Options for Patients with Type 1 Diabetes

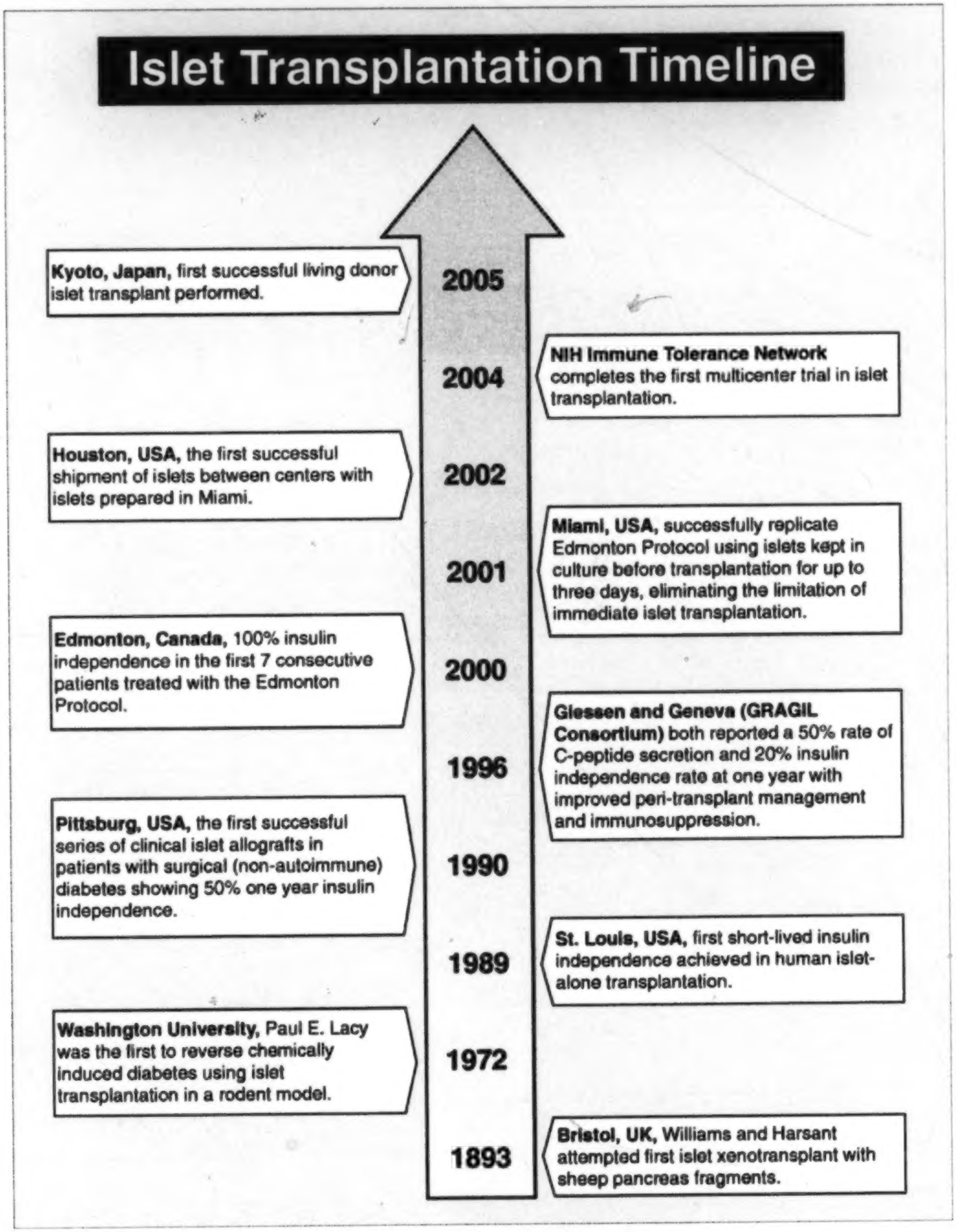
Diabetes is a disease that results from impaired glucose metabolism, either due to a complete loss of the insulin producing β-cells within the islets of Langerhans of the pancreas (Type 1 diabetes mellitus; "T1DM"), or due to a defect in insulin production and/or utilization (type 2 diabetes mellitus; "T2DM"). Currently, there are more than 200 million patients with diabetes worldwide, and it is projected that more than 5% of the world's adult population will be affected by diabetes by the year 2025 (1). T1DM is often referred to as juvenile-onset diabetes, as approximately 13,000 children are diagnosed with T1DM each year in the U.S., making T1DM the most prevalent chronic childhood disease (2). Since the discovery of insulin in 1921 by Drs. Banting, Best, and Collip, exogenous insulin injections have been the principle therapeutic option for patients with T1DM (3). Although careful blood glucose monitoring and insulin administration can provide patients with T1DM with a relatively good quality of life, the long term impact of this disease remain significant. For patients that are diagnosed with T1DM as children, it has been estimated that >15% will die before their 40th birthday, a mortality rate that is more than 20 times that of the general population (2). Causes of death related to T1DM include acute complications, such as hypoglycemic coma, and chronic secondary conditions, such as renal failure or cardiovascular disease (2). Patients with T1DM also face many chronic complications, including renal dysfunction, retinopathy, peripheral nephropathy, and gastroparesis, and they are more likely to also develop Celiac disease (2, 4). It has been determined that patients with T1DM represent 8% of those who are legally blind, 30% of all patients on dialysis due to end stage renal disease, and 20% of all patients receiving kidney transplants in the U.S. (2). In an effort to prevent these long term complications in patients with diabetes, the Diabetes Control and Complications Trial (DCCT) was conducted to examine the benefit of intensive blood glucose regulation by frequent insulin injection or pump (5-7). Results from the DCCT clearly demonstrated that this approach improved glycosylated hemoglobin levels and significantly protected against nephropathy, neuropathy, and retinopathy (6, 7). However, the penalty for improved glycemic control was a three fold increased risk of serious hypoglycemic events, including recurrent seizures and coma (6).

It has thus become clear that the restoration of an adequate islet mass would provide the best glucose regulation and long term health outcome for patients with T1DM. The first efforts directed at addressing this issue have involved whole pancreas transplantation, which was first described in 1967 (8). Since that time, more than 18,000 pancreas transplants have been performed, most often in the context of simultaneous cadaveric kidney and pancreas transplant for patients suffering from diabetes and end-stage renal disease (9). Since pancreas transplantation involves a major surgical procedure, potentially serious complications, and life-long immunosuppression, it has been primarily reserved for patients whose health status justifies the risk (i.e. end-stage renal disease or severe hypoglycemic unawareness). Even with recent improvements in the surgical procedure and management of patients following whole pancreas transplantation, only 50% of patients who have undergone pancreas-alone transplantation still present evidence of graft function at three years (9). Also, recent data from the United Network for Organ Sharing has shown that only 28% of the approximately 6,000 cadaveric pancreata donated each year are transplanted, since the organ must conform to strict donor criteria and requirements for short cold ischemic time to be considered suitable for transplantation (9, 10). Despite strong evidence that the procedure can prolong life, reverse established nephropathy, and improve quality of life, pancreas transplantation remains too limited and too morbid to advocate for most patients with T1DM, especially young patients who have not yet developed severe secondary complications (11-15).

1.2 History of Islet Transplantation

1.2.1 A Brief History of Islet Transplantation

Compared to solid organ transplantation, β-cell replacement via transplantation of isolated pancreatic islets presents a unique opportunity to reverse diabetes by implantation of a tissue without major surgery, vascular anastamoses, or drainage of the potent digestive enzymes produced by the exocrine pancreas, which most often causes the serious complications associated with whole pancreas transplantation (9). The first attempt to replace the insulin-producing cells in a patient with T1DM occurred in 1893, when physicians in Bristol transplanted fragments of a sheep's pancreas into a young boy suffering from diabetic ketoacidosis, although this graft of course failed in the absence of immunosuppression (16). After the discovery of insulin in 1921, it was thought that exogenous insulin replacement would be the answer for patients with T1DM, and therefore islet transplantation was not actively pursued. However, as more patients with T1DM began to live for many years with the disease, it became apparent that insulin replacement could not prevent the onset of severe secondary complications. Following the poor morbidity and mortality associated with the first series of whole pancreas transplants in the late 1960's, it became evident that isolated islet transplantation would be an attractive alternative (17). Dr. Paul Lacy at Washington University in St. Louis pioneered the first successful islet isolations and subsequent transplantation into chemically-induced diabetic rodents, which sparked an immediate interest in the implementation of clinical trials (18-21). Despite the successful reversal of diabetes in animal models of islet transplantation, clinical islet transplantation struggled for most of the 1970's and 1980's, since islet preparations were not purified and as such caused many serious complications following portal infusion, including portal vein thrombosis and portal hypertension (22). It was not until 1989 that Lacy's group finally achieved short-lived insulin independence in a patient with T1DM who received an islet graft following a previous kidney transplant (23). The group lead by Dr. Camillo Ricordi at the University of Pittsburgh reported the first series of clinical islet allografts that demonstrated improved insulin independence rates of 50% at one year (24). However, this result was only present in a cohort of patients who had become diabetic following surgical removal of their pancreas due to malignancies and could not be reproduced in patients with T1DM (24). In the late 1990's, the European GRAGIL consortium was the first to report modestly successful insulin independence rates of 20% at one year in patients with T1DM, due to improved peri-transplant management and immunosuppressive drug regimens (25). Since the results from Pittsburgh and the GRAGIL consortium were obtained in patients who had already undergone solid organ transplantation, there was no additional risk in terms of immunosuppression to the patients after receiving an islet graft (24, 25). However, after three decades of research, the one year insulin independence rates in clinical islet transplantation were still too low to justify the risks associated with portal infusion and life long immunosuppression in most patients with T1DM (14, 15, 25-27).



Islet Transplantation Timeline

2005

Kyoto, Japan, first successful living donor islet transplant performed.

2004

NIH Immune Tolerance Network completes the first multicenter trial in islet transplantation.

2002

Houston, USA, the first successful shipment of islets between centers with islets prepared in Miami.

2001

Miami, USA, successfully replicate Edmonton Protocol using islets kept in culture before transplantation for up to three days, eliminating the limitation of immediate islet transplantation.

2000

Edmonton, Canada, 100% insulin independence in the first 7 consecutive patients treated with the Edmonton Protocol.

1996

Giessen and Geneva (GRAGIL Consortium) both reported a 50% rate of C-peptide secretion and 20% insulin independence rate at one year with improved peri-transplant management and immunosuppression.

1990

Pittsburg, USA, the first successful series of clinical islet allografts in patients with surgical (non-autoimmune) diabetes showing 50% one year insulin independence.

1989

St. Louis, USA, first short-lived insulin independence achieved in human islet-alone transplantation.

1972

Washington University, Paul E. Lacy was the first to reverse chemically induced diabetes using islet transplantation in a rodent model.

1893

Bristol, UK, Williams and Harsant attempted first islet xenotransplant with sheep pancreas fragments.

Figure 1-1: Historical Timeline of Islet Transplantation.

(From Truong W, Shapiro AMJ. Islet Transplantation. In: Shackelford's Textbook of Surgery of the Alimentary Canal; 2005, In Press. Used with the authors' kind permission.)

1.2.2 The Edmonton Protocol: Clinical Success

A new protocol initiated by the Edmonton group in 1999 was designed for patients with 'brittle diabetes' who experienced extreme difficulty in managing their blood glucose levels ("glucose lability") and/or severe hypoglycemic unawareness (28). Compared to previous clinical islet transplantation studies, the Edmonton Protocol was unique in its avoidance of corticosteroids, use of potent immunosuppression with combined sirolimus, tacrolimus and anti-CD25 antibody to protect against rejection and recurrent autoimmunity, and use of two (or occasionally more) fresh islet preparations to provide a mean islet implant mass of approximately 13,000 islet equivalents (IE)/kg recipient body weight (28). For the first time, dramatic improvements in islet allograft survival were observed, and all of the first seven patients achieved sustained independence from insulin at one year (28). Since 2000, of 72 consecutive patients treated at the University of Alberta with recent modifications of the Edmonton Protocol, the one-year insulin independence rate remains steady at 82% after completed transplants (A.M.J. Shapiro, unpublished communication). An international multicentre trial (Immune Tolerance Network) has recently replicated the results obtained at the University of Alberta, but has shown a broad spectrum of success based on the centre's previous experience and skill in islet isolation and immunosuppressive management (29). The Miami group has demonstrated that islets can be cultured for up to three days prior to being transplanted, suggesting that islets can be manipulated and optimized ex vivo (30). They have also shown through collaboration with the Baylor group in Houston that islets can shipped to other centres, suggesting that the difficult process of islet isolation can be reserved for a few select centres, reducing variability in the preparations (30, 31). Based on these improved outcomes, the status of islet transplantation transitioned from 'research' to 'clinical funded acceptable care,' in 2001 in Alberta. The breakthrough data obtained in Edmonton has encouraged many centres around the world to implement clinical islet transplantation, and since 2000 more than 500 patients have been transplanted using the Edmonton Protocol or variations thereof (32).

Despite this success, islet transplantation remains restricted to patients with severe hypoglycemia or glycemic lability, and is presently unsuitable for the majority of patients with T1DM. Most patients require two or occasionally three islet implant procedures in order to achieve insulin independence, although insulin independence following single donor infusion has been reported in a small cohort of patients at one centre (33). While C-peptide secretion (>0.5ng/ml) has been maintained in 88% of islet graft recipients beyond three years in Edmonton, emerging data on the long-term insulin-independence rates have shown that after three years, only 50% of recipients remain off insulin, and at five years post-transplant this number falls to approximately 10% (A.M.J. Shapiro, unpublished communication). Multifactorial events including recurrent autoimmunity, sub-clinical allograft rejection, islet 'burn-out,' impairment of islet growth/replication in the presence of sirolimus and/or chronic islet toxicity from the immunosuppressive drugs likely account for the degradation in islet function, with disparity between sustained C-peptide secretion and loss of insulin independence over time. The risks associated with islet transplantation appear to increase with the number of infusions, and also with the total packed cell volume of cumulative grafts (34). The risk of branch vein thrombosis of the portal system or acute bleeding from the liver surface may increase if sequential islet infusions are required (34, 35). Although the risk of malignancy, post-transplant lymphoma and life-threatening sepsis has been very low in patients treated to date, fears of these complications limit a broader application in patients with less severe forms of diabetes including children. Moreover, a number of immunosuppression-related side-effects have been encountered, which can be dose or drug limiting in some patients (36). It is clear therefore that while outcomes have improved substantially after islet transplantation, extensive refinements in clinical protocols are needed both to improve safety and to enhance success with single donor islet infusions.

1.3 Mechanisms of Loss of Islet Mass in the Early Post-Transplant Period

1.3.1 Predicted β-cell Mass Necessary for Euglycemia

Before clinical islet transplantation can become more available to a wider population of patients with T1DM, the current requirement for a large number of donor islets must be overcome. Although it has been incredibly difficult to study directly in humans, careful examination of the T1DM disease process has suggested that an individual may be able to maintain euglycemia even after the loss of up to 90% of their β-cell mass, and beyond this threshold, the patient's glucose homestasis rapidly deteriorates, leading to overt diabetes (37). This hypothesis suggests that replacement of only a fraction of the endogenous islet mass may be sufficient to reverse diabetes. Indeed, surgical removal of up to 80% of the pancreas in patients with chronic pancreatitis or pancreatic malignancies rarely results in diabetes (Table 1-1), which supports the idea that only 10-20% of the estimated 10^6 endogenous islets may be necessary to maintain euglycemia in an individual (38).

Surgical Procedure	% with Diabetes Pre-Operation	% with Diabetes Post-Operation	% Mortality
Total Pancreatectomy (100%)	42	100	20.6
Near Total Pancreatectomy (80-95%)	40	100	1.3
Distal Pancreatectomy (40-80%)	17	32	1.8
Pancreaticoduodenectomy (50%)	15	26	0.6

Table 1-1: Incidence of Postoperative Diabetes Following Pancreatic Resection.

(Adapted from Slezak LA, Andersen DK. Pancreatic resection: effects on glucose metabolism. World J Surg 2001;25(4):452-60.)

So, in theory, one donor pancreas could possess enough islets to provide insulin independence in several patients. Despite marked improvements in islet isolation, including the Ricordi digestion chamber, controlled pancreatic distension with collagenase, and reduced endotoxin levels in purified enzyme blends, cadaveric islet isolations are at best only 20-50% efficient, due to factors such as hemodynamic instability in the donor, physiological changes during the process of brain death, cold ischemic time, and variation in enzymatic activities (39-43). Given the relative inefficiency of human islet isolations, one still might anticipate that insulin independence following single donor infusion would be routine, rather than the exception, since there should still be more than enough islets present to restore euglycemia. The fact that patients must receive >10,000 IE/kg to become insulin independent suggests that a large portion of the infused islets fail to engraft sufficiently to become functional (28, 29).

1.3.2 Transplanted vs. Functional β-cell Mass in Islet Transplantation

Islets only make up 1% of the pancreatic tissue and thus must be extracted from the donor organ. Unlike solid organ transplantation, where the donor tissue is delicately handled and maintained at 4 °C until being reperfused in a recipient, a pancreas selected for islet transplantation must be perfused with digestive enzyme, mechanically digested, and shaken for up to 30 min. at 37 °C (40, 44). The islets are then purified over a series of gradients and maintained in culture until transplantation. This process is extremely stressful to the islets, and the β-cells specifically are very sensitive and easily lost. When isolated human islets were evaluated immediately following the isolation procedure, it was determined that up to 30% of all islet cells stained TUNEL (terminal dUTP nick end labeling) positive for apoptosis, with the greatest proportion of the stained cells representing the β-cell population (45). Thus, some of the islet tissue is already in the process of dying even prior to portal vein infusion. When human islet grafts were examined following transplantation into immunodeficient nude mice, a dramatic decrease in islet mass was observed in the first two weeks after transplantation, resulting in a 70% decrease in β-cell mass by one month post-transplant (46). Even in murine models of islet transplantation where ideal, syngeneic donors can be selected, it has been determined that >60% of the islet graft is lost due to apoptosis within the first three days following implantation (47). The profound reduction in islet mass in this model has been found to be independent of the transplantation site (intraportal, subrenal capsular, or intrasplenic), suggesting that islets are inherently prone to post-transplant apoptosis (48). In clinical islet transplantation, it has been estimated that more than two thirds of the implanted mass fails to engraft, and following transplantation the β-cell functional mass continues to deteriorate moderately over time (36, 49). One might argue that the immediate functional demand on transplanted β-cells might account for their decreased survival, since the stress of engraftment combined with the need to respond to glucose stimulation may present too much of a burden. Although it has been determined that prolonged hyperglycemia post-transplant can lead to enhanced levels of islet apoptosis, examination of islet grafts in normoglycemic recipients has shown that a majority of the islet tissue is still apoptotic (47). Taken collectively, the fact that a majority of the islet mass is lost rapidly following transplantation into normoglycemic, syngeneic or immunocompromised donors strongly suggests that a significant pathophysiological stress results in the failure of transplanted islets to become established.

1.3.3 Hypoxia During Isolation and Following Implantation

In an intact pancreas, the islets are highly perfused; despite making up only 1% of the pancreatic mass, the islets receive up to 15% of the blood flow (50, 51). In fact, it has been shown that each cell within the islet is no more than one cell away from the arterial circulation (52). The intimate proximity of islet cells and blood flow makes sense from a functional standpoint, since this facilitates a rapid response by the islet cells, allowing their hormones to exert an effect quickly and maximally. The consequence of this is that β-cells have evolved to survive optimally when they are exposed to highly oxygenated blood (pO_2 of 40 mmHg) in nutrient rich surroundings (48). In whole pancreas transplantation, the majority of early graft loss and primary nonfunction can be attributed to vascular thrombosis, which suggests that adequate oxygenation is key for islet function and survival (53). Unfortunately, in islet transplantation there is no way to maintain islet oxygenation and perfusion at pre-isolation levels, since the current techniques of human islet isolation and purification cause the complete destruction of the islet capillary network (capillary network of intact islet shown in Fig. 1-2) (54, 55).

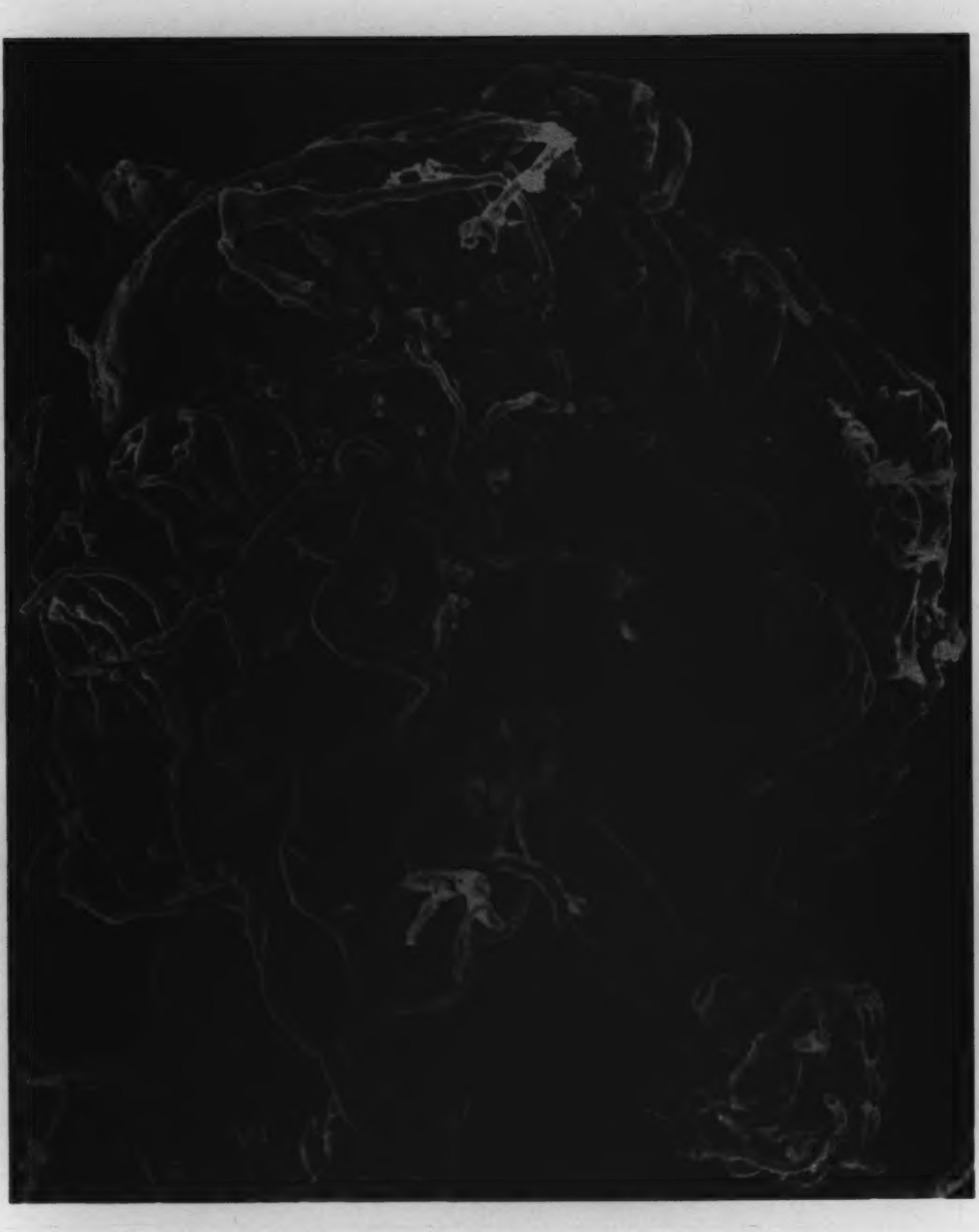


Figure 1-2: Scanning Electron Micrograph Illustrating the Dense Capillary Network of Pancreatic Islets.

(Adapted from Bonner-Weir S, Orci L. New perspectives on the microvasculature of the islets of Langerhans in the rat. Diabetes 1982;31(10):883-9. Image generously provided by and used with permission of S. Bonner-Weir.

When transplanted intraportally, the islets are embolized into the portal vein and its radicals, eventually settling into the hepatic presinusoidal capillaries (22). Since revascularization takes between 10-14 days, and potentially longer in patients with long-standing diabetes, during this period islets can only receive oxygen and nutrients via passive diffusion (48, 56-59). The islets are therefore exposed to an immediate hypoxic stress following transplantation, especially islets >200μm in diameter, which are nearly anoxic within the core (60, 61). Since the pO_2 of the portal circulation is typically 10-15 mmHg, there is already an oxygen deficit for the islet graft, which is magnified by the reduced ability of a devascularized islet to absorb oxygen uniformly (62, 63). This explains the observation that transplanted islets only possess a pO_2 of 8-10 mmHg, a fraction of the oxygen tension present in islets within an intact pancreas (48).

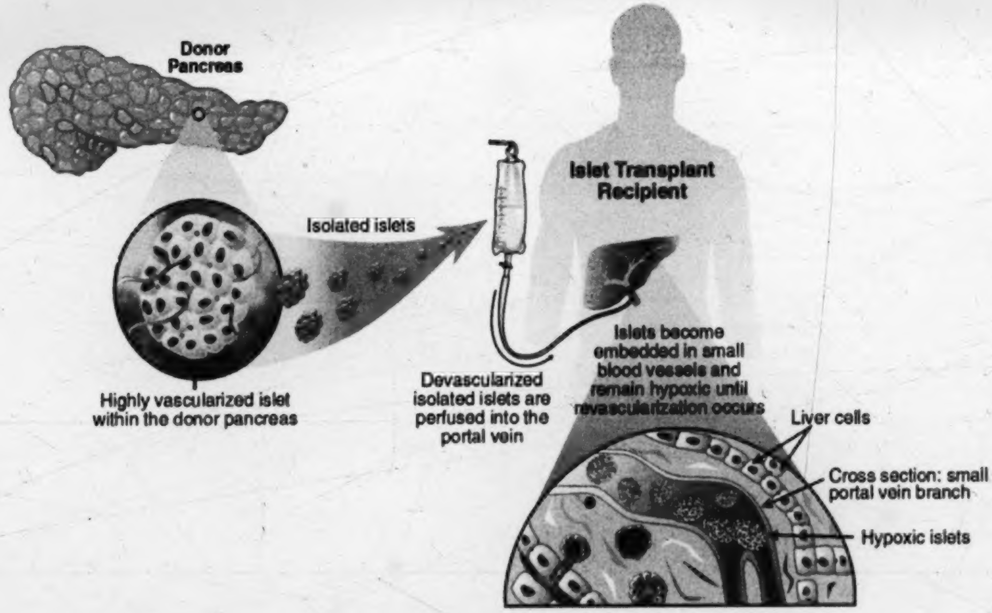

Donor Pancreas
Islet Transplant Recipient
Isolated islets
Highly vascularized islet within the donor pancreas
Devascularized isolated islets are perfused into the portal vein
Islets become embedded in small blood vessels and remain hypoxic until revascularization occurs
Liver cells
Cross section: small portal vein branch
Hypoxic islets

Figure 1-3: Causes of Hypoxia in Islet Transplantation.

1.3.4 Revascularization and Reperfusion Injury

As mentioned previously, it has been determined that transplanted islets generally require up to two weeks to become fully revascularized (54, 58, 64, 65). These reports are a little misleading however, since the degree of vascular density in transplanted islets was determined to be significantly reduced compared to pre-transplant levels (59). In fact, the vascular density of transplanted syngeneic rodent islets was determined to be less than 50% of that present in intact pancreatic islets at one month post-transplant, after which time the islets should be 'fully engrafted' (59). This observation was paralleled by the finding that the amount of blood flow within the transplanted islets was significantly reduced compared to pre-transplant levels, especially in diabetic recipients (59). Thus, the process of revascularization is slow and impaired for transplanted islets, even under optimized donor and recipient conditions.

Once revascularization has taken place, the ability of transplanted islets to absorb oxygen is immediately enhanced. However, following an extended period of up to two weeks in reduced oxygen conditions, the islet cells have adapted in order to compensate for the deficit. The rapid increase in oxygenated blood flow to any tissue inevitably leads to reperfusion injury and a burst of reactive oxygen species (ROS), including nitric oxide (NO), superoxide, peroxynitrite, hydrogen peroxide, etc. (reviewed in (66, 67)). It has long been known that β-cells are especially sensitive to ROS, both during the pathogenesis of T1DM and in the context of islet transplantation, leading to the rapid onset of β-cell loss of glucose responsiveness and death (68, 69). This is most likely related to the unusually low levels of several key antioxidant enzymes in islets, such as superoxide dismutase (SOD), Glutathione peroxidase (GPx), and catalase (70, 71). The impact of reperfusion-induced generation of ROS in islet transplantation has not been extensively investigated, which is most likely related to the difficulty in assessing the dynamic process and variable timing of revascularization in transplanted islets, compared to whole organ grafts which are directly anastamosed to the recipient's blood supply or ischemic injury studies where a certain vessel is clamped temporarily. Still, the well understood link between tissue reperfusion and ROS generation, as well as the extensively characterized sensitivity of β-cells to ROS, suggests that reperfusion injury may have a considerable impact on islet graft survival following transplantation.

1.3.5 Instant Blood-Mediated Inflammatory Reaction (IBMIR)

Recent data from the Uppsala Group have suggested that another process, termed the 'instant blood-mediated inflammatory reaction' (IBMIR), negatively influences islet engraftment and survival in the early post-transplant period. IBMIR was originally identified using an in vitro assay that detected a thrombotic reaction between human islets and heterologous, ABO-compatible blood (72). Further investigation into the mechanism of IBMIR has shown that islets naturally express Tissue Factor, a protein which acts as a receptor and cofactor for Factor VII, leading to coagulation (73). Following the stressful process of isolation, human islets have been shown to release Tissue Factor along with glucagon and insulin, which in turn upregulates thrombin, leading to platelet activation and binding to the surface of the islets. This ultimately causes to the formation of a fibrin capsule around the islet and disruption of the islet morphology (72-74). Although most of this process has been characterized using an in vitro tubing loop model, examination of serum in patients undergoing islet transplantation has shown that a statistically significant increase in the serum concentration of thrombin/anti-thrombin complexes is present almost immediately following portal infusion, with peak levels occurring at 15 min., even when there was no clinical evidence of portal hypertension or intraportal thrombosis (73). The direct impact of IBMIR on early loss of islet function and mass has yet to be fully characterized. However, given that platelet activation is one of the primary contributing factors in the generation of an inflammatory response, IBMIR is most likely one of the key early processes in islet transplantation that elicits an immune response, characterized by macrophage-mediated islet destruction and enhancement of allo-antigen driven immunological attack (73, 75).

1.4 Other Factors Influencing Long Term Islet Graft Survival

As is the case in all allogeneic organ transplantation, islet grafts are subject to allo-rejection, characterized by enhanced local expression of chemokines, secretion of proinflammatory cytokines by infiltrating immune cells, and direct killing by activated T-cells. Chemokines play a critical role in the recruitment, activation, and migration of mononuclear cells during inflammation. Compared to syngeneic islet grafts, which exhibit little evidence of chemokines or their associated receptors, rodent islet allografts have been shown to express high levels of CCR5, CCR2, and CXCR3 within the first week post-transplant (76). It has also been determined through transplantation of allogeneic rodent islet grafts into CCR5$^{-/-}$ recipients that this chemokine plays an important role in mediating long-term graft survival (76). The extreme sensitivity of β-cells to the proinflammatory cytokines interleukin-1β (IL-1β), interferon-γ (IFNγ), and tumor necrosis factor-α (TNFα) has long been considered one of the primary contributing factors in the pathogenesis of T1DM, and the production of these cytokines following monocyte and T-cell infiltration in islet grafts is no less destructive (reviewed in (75)).

One unique component of islet transplantation in patients with T1DM is the possibility of recurrent autoimmunity, which may elevate the demand for immunosuppression. Indeed, it has been well established using a rodent model of T1DM, the non-obese diabetic (NOD) mouse, that control of recurrent autoimmune reactivity to β-cells is one of the most difficult obstacles to overcome in islet transplantation (reviewed in (77, 78)). Although it has been quite difficult to study recurrent autoimmunity in clinical patients, some evidence exists to suggest that levels of autoantibodies to GAD (glutamic acid decarboxylase) and IA-2 increase following islet transplantation, although the direct impact of this phenomenon on graft survival is not yet clear (79, 80). If recurrent autoimmunity does alter immunosuppressive drug functional thresholds, this presents yet another problem in the context of islet transplantation, as many of the drugs are directly β-cell toxic. In fact, up to 15% of non-diabetic patients who receive solid organ grafts can develop post-transplant diabetes as a result of calcineurin inhibitor therapy (i.e. tacrolimus) or steroids (i.e. prednisone) (81, 82). One of the main reasons why the Edmonton Protocol was successful involved the limited use of β-cell toxic immunosuppressive drugs, instead using antibody induction followed by reduced doses of tacrolimus, rapamycin (Sirolimus) and now mycophenolate mofetil (MMF) (28). However, most patients that are candidates for the Edmonton Protocol have had disregulated diabetes for many years, and as such their renal status is somewhat impaired (28). This leads to an increased sensitivity to the deleterious renal side effects of these immunosuppressive drugs, and thus limits the extent to which the dose can be increased to preserve graft function (83). It is therefore likely that immunosuppressive drugs either contribute directly to β-cell loss over time via toxicity, or indirectly by incomplete protection against recurrent autoimmunity and/or alloreactivity.

1.5 Conclusions

β-cell replacement through islet transplantation presents the best opportunity to treat T1DM and prevent the long term serious complications associated with this disease. However, the current requirement for islets derived from two or more cadaveric donors severely limits the availability of this procedure. The process of engraftment is especially difficult for transplanted islets, due to prolonged hypoxia and subsequent reperfusion injury during the process of revascularization, which takes up to two weeks. During this period, the survival of the islets is also negatively influenced by the IBMIR process and resulting inflammation and recruitment of the immune system. Strategies targeted at preserving β-cell mass during the engraftment phase should have a significant and immediate impact on islet transplantation by reducing the amount of islet tissue necessary to reverse diabetes. In this regard, islet transplantation could be made available to a broader population of patients with T1DM.

1.6 References

1. King H, Aubert RE, Herman WH. Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. Diabetes Care 1998;21(9):1414-31.

2. National Diabetes Data Group (U.S.), National Institute of Diabetes and Digestive and Kidney Diseases (U.S.), National Institutes of Health (U.S.). Diabetes in America. 2nd ed. [Bethesda, Md.]: National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases; 1995.

3. Bliss M. The discovery of insulin. 3rd pbk. ed. Toronto: University of Toronto Press; 2000.

4. Schuppan D, Hahn EG. Celiac disease and its link to type 1 diabetes mellitus. J Pediatr Endocrinol Metab 2001;14 Suppl 1:597-605.

5. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. N Engl J Med 1993;329(14):977-86.

6. Keen H. The Diabetes Control and Complications Trial (DCCT). Health Trends 1994;26(2):41-3.

7. Diabetes Control and Complications Trial (DCCT). Update. DCCT Research Group. Diabetes Care 1990;13(4):427-33.

8. Kelly WD, Lillehei RC, Merkel FK, Idezuki Y, Goetz FC. Allotransplantation of the pancreas and duodenum along with the kidney in diabetic nephropathy. Surgery 1967;61(6):827-37.

9. Larsen JL. Pancreas transplantation: indications and consequences. Endocr Rev 2004;25(6):919-46.

10. Statistical Data Reported by the U.S. Scientific Registry of Transplant Recipients and the Organ Procurement and Transplantation Network. In; 2005.

11. Fioretto P, Steffes MW, Sutherland DE, Goetz FC, Mauer M. Reversal of lesions of diabetic nephropathy after pancreas transplantation. N Engl J Med 1998;339(2):69-75.

12. Tyden G, Tollemar J, Bolinder J. Combined pancreas and kidney transplantation improves survival in patients with end-stage diabetic nephropathy. Clin Transplant 2000;14(5):505-8.

13. Reddy KS, Stablein D, Taranto S, Stratta RJ, Johnston TD, Waid TH, et al. Long-term survival following simultaneous kidney-pancreas transplantation

versus kidney transplantation alone in patients with type 1 diabetes mellitus and renal failure. Am J Kidney Dis 2003;41(2):464-70.

14. Secchi A, Di Carlo V, Martinenghi S, La Rocca E, Caldara R, Spotti D, et al. Effect of pancreas transplantation on life expectancy, kidney function and quality of life in uraemic type 1 (insulin-dependent) diabetic patients. Diabetologia 1991;34 Suppl 1:S141-4.

15. Gross CR, Limwattananon C, Matthees BJ. Quality of life after pancreas transplantation: a review. Clin Transplant 1998;12(4):351-61.

16. Williams P. Notes on diabetes treated with extract and by grafts of sheep's pancreas. BMJ 1894;2:1303-4.

17. Sutherland DE, Gruessner RW, Dunn DL, Matas AJ, Humar A, Kandaswamy R, et al. Lessons learned from more than 1,000 pancreas transplants at a single institution. Ann Surg 2001;233(4):463-501.

18. Lacy PE, Kostianovsky M. Method for the isolation of intact islets of Langerhans from the rat pancreas. Diabetes 1967;16(1):35-9.

19. Ballinger WF, Lacy PE. Transplantation of intact pancreatic islets in rats. Surgery 1972;72(2):175-86.

20. Reckard CR, Ziegler MM, Barker CF. Physiological and immunological consequences of transplanting isolated pancreatic islets. Surgery 1973;74(1):91-9.

21. Kemp CB, Knight MJ, Scharp DW, Ballinger WF, Lacy PE. Effect of transplantation site on the results of pancreatic islet isografts in diabetic rats. Diabetologia 1973;9(6):486-91.

22. Walsh TJ, Eggleston JC, Cameron JL. Portal hypertension, hepatic infarction, and liver failure complicating pancreatic islet autotransplantation. Surgery 1982;91(4):485-7.

23. Scharp DW, Lacy PE, Santiago JV, McCullough CS, Weide LG, Falqui L, et al. Insulin independence after islet transplantation into type I diabetic patient. Diabetes 1990;39(4):515-8.

24. Ricordi C, Tzakis AG, Carroll PB, Zeng YJ, Rilo HL, Alejandro R, et al. Human islet isolation and allotransplantation in 22 consecutive cases. Transplantation 1992;53(2):407-14.

25. Benhamou PY, Oberholzer J, Toso C, Kessler L, Penfornis A, Bayle F, et al. Human islet transplantation network for the treatment of Type I diabetes: first data from the Swiss-French GRAGIL consortium (1999-2000). Groupe de

Recherche Rhin Rhjne Alpes Geneve pour la transplantation d'Ilots de Langerhans. Diabetologia 2001;44(7):859-64.

26. Brendel M HB, Schulz A, Bretzel R. International Islet Transplant Registry Report. University of Giessen, Germany 2001:1.

27. Hering B RC. Islet transplantation for patients with Type 1 diabetes: results, research priorities, and reasons for optimism. Graft 1999;2(1):12.

28. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000;343(4):230-8.

29. Shapiro AM, Ricordi C, Hering B. Edmonton's islet success has indeed been replicated elsewhere. Lancet 2003;362(9391):1242.

30. Goss JA, Schock AP, Brunicardi FC, Goodpastor SE, Garber AJ, Soltes G, et al. Achievement of insulin independence in three consecutive type-1 diabetic patients via pancreatic islet transplantation using islets isolated at a remote islet isolation center. Transplantation 2002;74(12):1761-6.

31. Goss JA, Goodpastor SE, Brunicardi FC, Barth MH, Soltes GD, Garber AJ, et al. Development of a human pancreatic islet-transplant program through a collaborative relationship with a remote islet-isolation center. Transplantation 2004;77(3):462-6.

32. International Islet Transplant Registry. In; 2005.

33. Hering BJ, Kandaswamy R, Ansite JD, Eckman PM, Nakano M, Sawada T, et al. Single-donor, marginal-dose islet transplantation in patients with type 1 diabetes. Jama 2005;293(7):830-5.

34. Casey JJ, Lakey JR, Ryan EA, Paty BW, Owen R, O'Kelly K, et al. Portal venous pressure changes after sequential clinical islet transplantation. Transplantation 2002;74(7):913-5.

35. Bucher P, Mathe Z, Bosco D, Becker C, Kessler L, Greget M, et al. Morbidity associated with intraportal islet transplantation. Transplant Proc 2004;36(4):1119-20.

36. Ryan EA, Lakey JR, Paty BW, Imes S, Korbutt GS, Kneteman NM, et al. Successful islet transplantation: continued insulin reserve provides long-term glycemic control. Diabetes 2002;51(7):2148-57.

37. Weir GC, Bonner-Weir S. Five stages of evolving beta-cell dysfunction during progression to diabetes. Diabetes 2004;53 Suppl 3:S16-21.

38. Slezak LA, Andersen DK. Pancreatic resection: effects on glucose metabolism. World J Surg 2001;25(4):452-60.

39. Ricordi C, Lacy PE, Finke EH, Olack BJ, Scharp DW. Automated method for isolation of human pancreatic islets. Diabetes 1988;37(4):413-20.

40. Lakey JR, Warnock GL, Shapiro AM, Korbutt GS, Ao Z, Kneteman NM, et al. Intraductal collagenase delivery into the human pancreas using syringe loading or controlled perfusion. Cell Transplant 1999;8(3):285-92.

41. Linetsky E, Bottino R, Lehmann R, Alejandro R, Inverardi L, Ricordi C. Improved human islet isolation using a new enzyme blend, liberase. Diabetes 1997;46(7):1120-3.

42. Ricordi C, Lakey JR, Hering BJ. Challenges toward standardization of islet isolation technology. Transplant Proc 2001;33(1-2):1709.

43. Contreras JL, Eckstein C, Smyth CA, Sellers MT, Vilatoba M, Bilbao G, et al. Brain death significantly reduces isolated pancreatic islet yields and functionality in vitro and in vivo after transplantation in rats. Diabetes 2003;52(12):2935-42.

44. Tsujimura T, Kuroda Y, Avila JG, Kin T, Oberholzer J, Shapiro AM, et al. Influence of pancreas preservation on human islet isolation outcomes: impact of the two-layer method. Transplantation 2004;78(1):96-100.

45. Paraskevas S, Maysinger D, Wang R, Duguid TP, Rosenberg L. Cell loss in isolated human islets occurs by apoptosis. Pancreas 2000;20(3):270-6.

46. Davalli AM, Ogawa Y, Ricordi C, Scharp DW, Bonner-Weir S, Weir GC. A selective decrease in the beta cell mass of human islets transplanted into diabetic nude mice. Transplantation 1995;59(6):817-20.

47. Biarnes M, Montolio M, Nacher V, Raurell M, Soler J, Montanya E. Beta-cell death and mass in syngeneically transplanted islets exposed to short- and long-term hyperglycemia. Diabetes 2002;51(1):66-72.

48. Carlsson PO, Palm F, Andersson A, Liss P. Markedly decreased oxygen tension in transplanted rat pancreatic islets irrespective of the implantation site. Diabetes 2001;50(3):489-95.

49. Rickels MR, Schutta MH, Markmann JF, Barker CF, Naji A, Teff KL. {beta}-Cell function following human islet transplantation for type 1 diabetes. Diabetes 2005;54(1):100-6.

50. Jansson L, Hellerstrom C. Stimulation by glucose of the blood flow to the pancreatic islets of the rat. Diabetologia 1983;25(1):45-50.

51. Bonner-Weir S, Orci L. New perspectives on the microvasculature of the islets of Langerhans in the rat. Diabetes 1982;31(10):883-9.

52. Lifson N, Kramlinger KG, Mayrand RR, Lender EJ. Blood flow to the rabbit pancreas with special reference to the islets of Langerhans. Gastroenterology 1980;79(3):466-73.

53. Drachenberg CB, Papadimitriou JC, Farney A, Wiland A, Blahut S, Fink JC, et al. Pancreas transplantation: the histologic morphology of graft loss and clinical correlations. Transplantation 2001;71(12):1784-91.

54. Menger MD, Jaeger S, Walter P, Feifel G, Hammersen F, Messmer K. Angiogenesis and hemodynamics of microvasculature of transplanted islets of Langerhans. Diabetes 1989;38 Suppl 1:199-201.

55. Stagner JI, Mokshagundam S, Samols E. Hormone secretion from transplanted islets is dependent upon changes in islet revascularization and islet architecture. Transplant Proc 1995;27(6):3251-4.

56. Jansson L, Sandler S. Influence of hyperglycemia on blood perfusion of autotransplanted pancreatic islets in diabetic rats. Diabetes 1989;38 Suppl 1:196-8.

57. Menger MD, Vajkoczy P, Leiderer R, Jager S, Messmer K. Influence of experimental hyperglycemia on microvascular blood perfusion of pancreatic islet isografts. J Clin Invest 1992;90(4):1361-9.

58. Sandberg JO, Margulis B, Jansson L, Karlsten R, Korsgren O. Transplantation of fetal porcine pancreas to diabetic or normoglycemic nude mice. Evidence of a rapid engraftment process demonstrated by blood flow and heat shock protein 70 measurements. Transplantation 1995;59(12):1665-9.

59. Carlsson PO, Palm F, Mattsson G. Low revascularization of experimentally transplanted human pancreatic islets. J Clin Endocrinol Metab 2002;87(12):5418-23.

60. Dionne KE, Colton CK, Yarmush ML. Effect of hypoxia on insulin secretion by isolated rat and canine islets of Langerhans. Diabetes 1993;42(1):12-21.

61. Giuliani M, Moritz W, Bodmer E, Dindo D, Kugelmeier P, Lehmann R, et al. Central necrosis in isolated hypoxic human pancreatic islets: evidence for postisolation ischemia. Cell Transplant 2005;14(1):67-76.

62. Pileggi A, Ricordi C, Alessiani M, Inverardi L. Factors influencing Islet of Langerhans graft function and monitoring. Clin Chim Acta 2001;310(1):3-16.

63. Sezai S, Sakurabayashi S, Yamamoto Y, Morita T, Hirano M, Oka H. Hepatic arterial and portal venous oxygen content and extraction in liver cirrhosis. Liver 1993;13(1):31-5.

64. Andersson A, Korsgren O, Jansson L. Intraportally transplanted pancreatic islets revascularized from hepatic arterial system. Diabetes 1989;38 Suppl 1:192-5.

65. Mendola JF, Goity C, Fernandez-Alvarez J, Saenz A, Benarroch G, Fernandez-Cruz L, et al. Immunocytochemical study of pancreatic islet revascularization in islet isograft. Effect of hyperglycemia of the recipient and of in vitro culture of islets. Transplantation 1994;57(5):725-30.

66. Becker LB. New concepts in reactive oxygen species and cardiovascular reperfusion physiology. Cardiovasc Res 2004;61(3):461-70.

67. Li C, Jackson RM. Reactive species mechanisms of cellular hypoxia-reoxygenation injury. Am J Physiol Cell Physiol 2002;282(2):C227-41.

68. Nomikos IN, Wang Y, Lafferty KJ. Involvement of O2 radicals in 'autoimmune' diabetes. Immunol Cell Biol 1989;67 (Pt 1):85-7.

69. Stevens RB, Ansite JD, Mills CD, Lokeh A, Rossini TJ, Saxena M, et al. Nitric oxide mediates early dysfunction of rat and mouse islets after transplantation. Transplantation 1996;61(12):1740-9.

70. Tiedge M, Lortz S, Drinkgern J, Lenzen S. Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. Diabetes 1997;46(11):1733-42.

71. Lenzen S, Drinkgern J, Tiedge M. Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. Free Radic Biol Med 1996;20(3):463-6.

72. Bennet W, Sundberg B, Groth CG, Brendel MD, Brandhorst D, Brandhorst H, et al. Incompatibility between human blood and isolated islets of Langerhans: a finding with implications for clinical intraportal islet transplantation? Diabetes 1999;48(10):1907-14.

73. Moberg L, Johansson H, Lukinius A, Berne C, Foss A, Kallen R, et al. Production of tissue factor by pancreatic islet cells as a trigger of detrimental thrombotic reactions in clinical islet transplantation. Lancet 2002;360(9350):2039-45.

74. Ozmen L, Ekdahl KN, Elgue G, Larsson R, Korsgren O, Nilsson B. Inhibition of thrombin abrogates the instant blood-mediated inflammatory reaction triggered by isolated human islets: possible application of the thrombin inhibitor melagatran in clinical islet transplantation. Diabetes 2002;51(6):1779-84.

75. Rabinovitch A, Suarez-Pinzon WL. Cytokines and their roles in pancreatic islet beta-cell destruction and insulin-dependent diabetes mellitus. Biochem Pharmacol 1998;55(8):1139-49.

76. Abdi R, Means TK, Luster AD. Chemokines in islet allograft rejection. Diabetes Metab Res Rev 2003;19(3):186-90.

77. Rossini AA, Mordes JP, Greiner DL, Stoff JS. Islet cell transplantation tolerance. Transplantation 2001;72(8 Suppl):S43-6.

78. Pearson T, Markees TG, Serreze DV, Pierce MA, Wicker LS, Peterson LB, et al. Islet cell autoimmunity and transplantation tolerance: two distinct mechanisms? Ann N Y Acad Sci 2003;1005:148-56.

79. Jaeger C, Brendel MD, Eckhard M, Bretzel RG. Islet autoantibodies as potential markers for disease recurrence in clinical islet transplantation. Exp Clin Endocrinol Diabetes 2000;108(5):328-33.

80. Bosi E, Braghi S, Maffi P, Scirpoli M, Bertuzzi F, Pozza G, et al. Autoantibody response to islet transplantation in type 1 diabetes. Diabetes 2001;50(11):2464-71.

81. Jindal RM, Sidner RA, Milgrom ML. Post-transplant diabetes mellitus. The role of immunosuppression. Drug Saf 1997;16(4):242-57.

82. Djamali A, Premasathian N, Pirsch JD. Outcomes in kidney transplantation. Semin Nephrol 2003;23(3):306-16.

83. Ryan EA, Paty BW, Senior PA, Shapiro AM. Risks and side effects of islet transplantation. Curr Diab Rep 2004;4(4):304-9.

Chapter 2

Interventional Strategies to Prevent β-cell Apoptosis in Islet Transplantation

2.1 Introduction

The Edmonton Protocol has clearly demonstrated that islet transplantation can provide a population of patients with T1DM with excellent glycemic control and often insulin independence, but the current necessity for >10,000 IE/kg recipient body weight limits the availability of this procedure (1). Investigation into the pathogenesis of T1DM and the relatively low incidence of diabetes following partial pancreatectomy has suggested that only a portion, perhaps as little as 20% of the islets within a pancreas are necessary to maintain euglycemia (2, 3). Since many more islets must be transplanted in order to reverse diabetes, a significant portion of the transplanted islets must fail to engraft properly. In fact, in both animal models and clinical islet transplantation, it has been determined that up to 70% of the transplanted β-cell mass never becomes functional (4-6). Since this profound loss has been observed in both immunodeficient and syngeneic islet transplantation models, the biggest factor that negatively influences islet survival must be a non-immune mediated physiological stress, namely prolonged hypoxia and gradual reperfusion during the revascularization process, which can take up to two weeks (7-10). Tissue Factor expression and release in isolated islets negatively influences the engraftment of transplanted islets, since it causes the IBMIR and subsequent platelet activation, clot formation, and lymphocyte recruitment (11, 12). Also, local secretion of pro-inflammatory cytokines by infiltrating lymphocytes further contributes to islet demise. For all of these reasons, a significant fraction of the islet graft becomes nonfunctional rapidly after portal infusion and is lost due to apoptosis, which begins during the isolation process, peaks 2-3 days post-transplant, and continues for approximately two weeks, until what remains of the graft has stabilized and become revascularized (5, 6, 8, 10, 13-15). Given the limited supply of cadaveric donor pancreata and the prevalence of T1DM, considerable efforts have been made to prevent the apoptotic loss of islet mass in the immediate post-transplant period. Widespread apoptosis in the implanted tissue may also have long term deleterious consequences in islet transplantation, since the recipient's immune system is challenged with a large amount of apoptotic tissue, possessing both allo- and auto-antigens from two or more donors. This suggests that preservation of islet graft function with long-term immunosuppression or tolerance induction is a major obstacle in the clinical setting.

2.2 Pathways to Caspase Activation and Apoptosis

Higher order organisms must regulate both the quality and quantity of each cell type they possess in order to prevent the deleterious effects associated with overgrowth, mutation, infection, or damage. This evolutionarily conserved mechanism is termed apoptosis, or "programmed cell death", and there are multiple triggers and pathways that control its initiation and progress within the cell. At the heart of this mechanism lies the caspases, a set of highly conserved cysteine proteases that are activated by a variety of pro-apoptotic stimuli. All caspases reside in the cell as zymogens, which become activated following proteolytic cleavage of the N-terminal prodomain (16). Caspases are activated in a hierarchical order, where initiator caspases (i.e. caspases- 8, and 10) function to cleave effector caspases (i.e. caspases- 3 and 7), which in turn degrade a number of intracellular protein substrates. This leads to the classical morphological changes associated with apoptosis, including chromatin condensation, nuclear degeneration, and cellular dehydration (16).

While an organism grows, it must monitor both its extracellular environment and ongoing internal processes in order to establish the suitability of progressing through the cell cycle. Similar in that respect to mitosis, apoptosis can be initiated by certain environmental cues, whether internal or external, to prevent the disruption of homeostasis. Once apoptosis has begun, it is quite difficult to reverse, so the cellular machinery has two distinct types of signaling cascades, each of which has multiple points where apoptotic effector molecules can act to either accelerate or terminate the sequence of events that will ultimately lead to cell death.

Extracellular events present during the inflammatory response, such as the release of cytokines including TNFα, IL-1β, and IFN-γ by infiltrating leukocytes or direct cytotoxic T-lymphocyte engagement can initiate apoptosis. These 'extrinsic' cues function via surface molecules in the death receptor pathway, where specific ligand-receptor binding (such as TNF–TNF Receptor I binding, Fas (CD95)-Fas Ligand (CD178) binding, etc.) leads to receptor clustering, adapter molecule recruitment (i.e. Fas-associated Death Domain or 'FADD'), and formation of the death-inducing signaling complex (DISC) (17). Caspase-8 associates with the DISC complex, where it is activated and released, leading to effector caspase activation (notably caspase-3) (Figure 2-1) (16, 17).

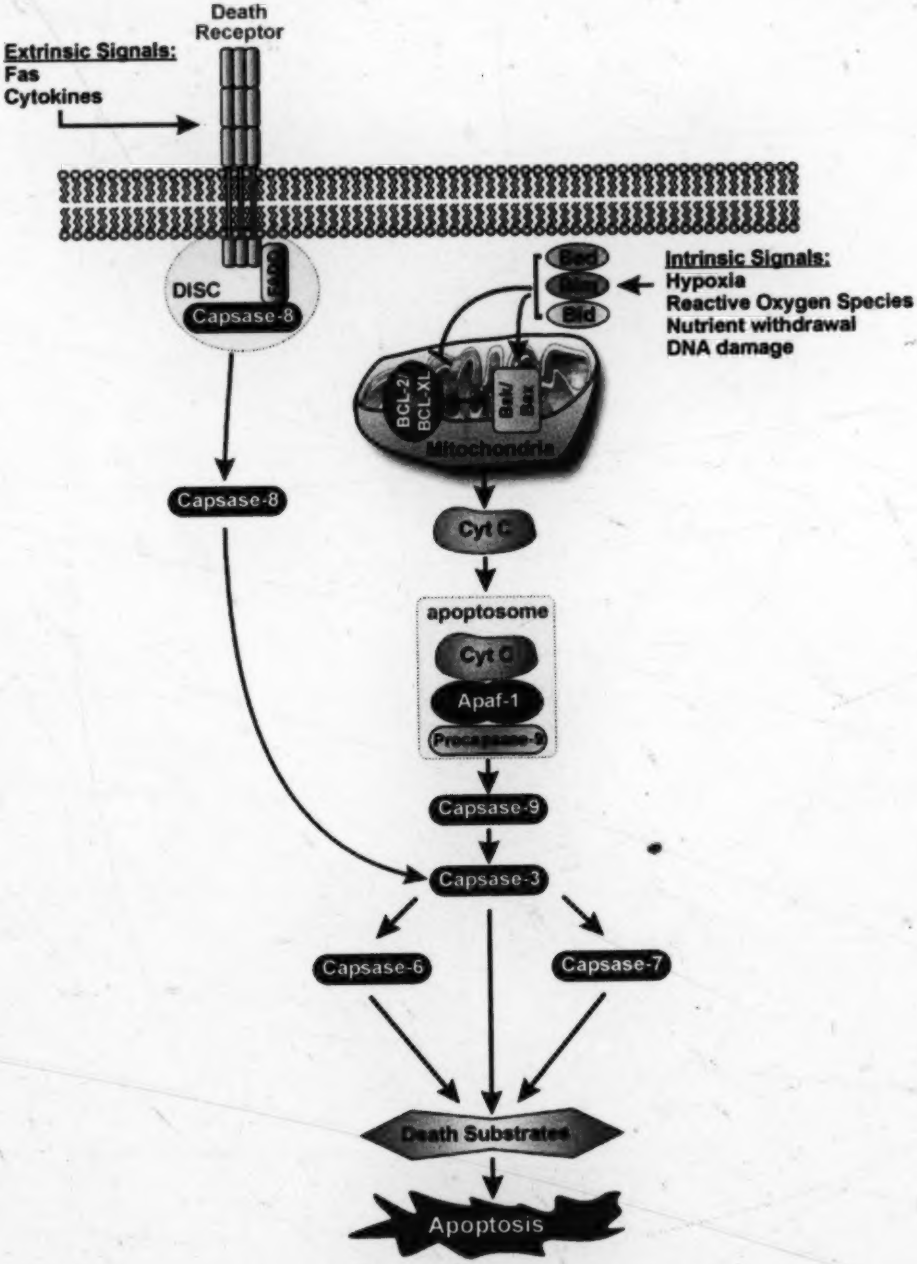


Figure 2-1: Summary of extrinsic and intrinsic pathways to apoptosis.

Intracellular or 'intrinsic' cues, such as DNA damage, hypoxia, nutrient deprivation, or ROS function via the mitochondrial pathway, which is tightly modulated by the BCL-2 proteins. In a healthy cell, pro-apoptotic BCL-2 proteins (Bim, Bid, Bad, Bax, Bak) are present in their inactive form, while anti-apoptotic BCL-2 proteins (BCL-2, BCL-XL) are constitutively active and reside in the outer membrane of the mitochondria (18). Following an intrinsic cue, pro-apoptotic BCL-2 proteins become activated and translocate to the mitochondria, where they either bind to and inactivate anti-apoptotic BCL-2 proteins or form pores in the mitochondrial membrane, which facilitates the release of cytochrome c into the cytosol. Once cytochrome c accumulates in the cytosol, it complexes with procaspase-9 and Apaf-1 to form the 'apoptosome', which in turn activates caspase-3 (Figure 2-1). Thus, the default BCL-2 signal is to preserve mitochondrial membrane integrity and prevent apoptosis, and as such apoptosis can only occur when the concentration of pro-apoptotic BCL-2 proteins exceeds that of the anti-apoptotic BCL-2 proteins at the mitochondrial membrane.

Originally named for a B-cell lymphoma gene, the BCL-2 family has been separated into three subclasses: pro-apoptotic initiators, pro-apoptotic effectors, and anti-apoptotic mediators. The initiator BCL-2 members are characterized by a single BCL-2 homology-3 (BH3) domain and are regulated by a variety of post-translational modifications following pro-apoptotic stimuli. Bim, Bad, and Bid are sequestered in the cytoskeleton (Bim) or cytosol (Bad and Bid) of healthy cells, and following a pro-apoptotic signal, they translocate to the mitochondria where they bind to and inhibit the anti-apoptotic BCL-2 family proteins BCL-2 and BCL-

XL (18). Effector pro-apoptotic members (i.e. Bax and Bak) possess three BH3 domains and reside in their inactive form in the cytosol (Bax) or loosely attached to mitochondrial membranes (Bak) (18). Following a pro-apoptotic signal, initiator BCL-2 proteins cause the allosteric activation and oligomerization of Bax and Bak, which allows them to form pores in the outer mitochondrial membrane, leading to cytochrome c release (18). It should be noted that the mitochondrial pathway can be indirectly triggered through less perspicuous extracellular signals. For example, following death receptor ligation, activated caspase-8 can cleave and activate Bid (18). Both intracellular and extracellular signaling cascades converge at the point of caspase-3 activation, which is often considered the "point of no return" in apoptosis (reviewed in (16, 19)).

2.3 Genetic Manipulations to Prevent β-cell Apoptosis

2.3.1 Inhibition of Extrinsic Signals

T1DM occurs following the selective loss of pancreatic islets, which is characterized by mononuclear cell infiltration around and within the islets, a histological feature termed 'insulitis'. This peri-islet inflammation is associated with pro-inflammatory cytokine (i.e. IL-1β, TNFα, IFNγ) release by monocytes and Fas ligation by autoreactive T-cells, leading to the destruction of the β-cells and the onset of hyperglycemia (20). Since the sensitivity of islets to cytokine and Fas-mediated apoptosis during the process of autoimmunity is well established, and since the allo-immune response exerts a similar effect on transplanted tissue, some of the earliest efforts in prevention of β-cell death following transplantation have involved the inhibition of these extrinsic signals.

Apoptosis via Fas/FasL interactions has been proposed to be a major T-cell mediated effector mechanism in the pathogenesis of T1DM. Examination of Fas expression in transplanted syngeneic islets from NOD mice demonstrated that autoimmune infiltration of islet grafts leads to high Fas levels in β-cells (21). In addition, it has been shown that proinflammatory cytokine exposure leads to enhanced Fas expression in both murine and human islets (21, 22). As mentioned previously, Fas signaling leads to FADD recruitment, DISC formation, and caspase-8 activation. Similarly, pro-inflammatory cytokines bind to their receptors on β-cells, leading to apoptosis via FADD recruitment and caspase-8 activation. In the mid-1990's, an endogenous inhibitor of caspase-8 activation was identified, termed the cellular FLICE-inhibitory protein (cFLIP) (FLICE is another name for caspase-8) (23). cFLIP is the same length as and structurally similar to caspase-8, but its caspase domain is altered, rendering it enzymatically inactive. Thus, cFLIP functions as a dominant-negative inhibitor of caspase-8 at the DISC, preventing the activation of caspase-8 and its ability to activate downstream effector caspases (Figure 2-2).

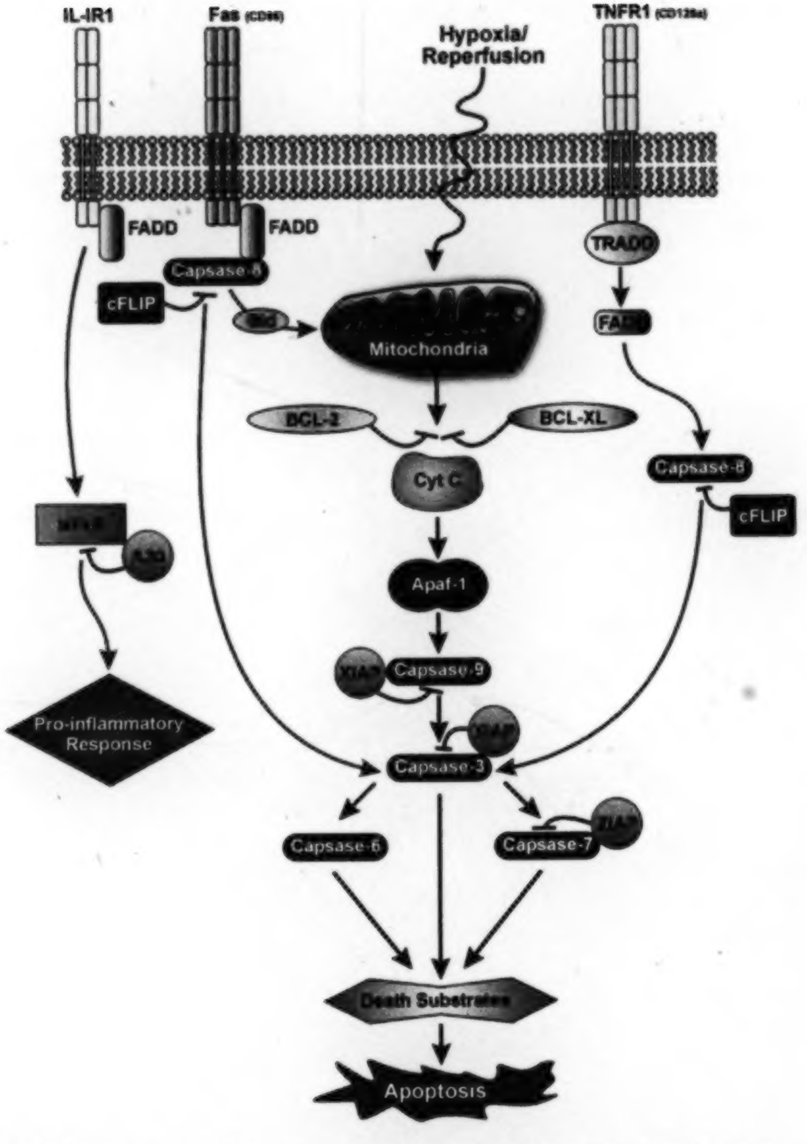


Figure 2-2: Summary of anti-apoptotic gene manipulations in islet transplantation.

Genetic manipulations targeted to block initiation of apoptotic pathways (i.e. cFLIP, BCL-2, and BCL-XL) have proven effective in preventing apoptosis induced by their respective targets in vitro, but have failed to show any significant protective effect in vivo during islet engraftment. XIAP has potential to be a potent inhibitor of islet death in vitro and in vivo, most likely due to its ability to prevent activation of effector caspases that function late in apoptosis, beyond the convergence point of many different stimuli.

Given the attractive anti-apoptotic function of cFLIP, the protective effect of cFLIP overexpression in β-cells during islet transplantation has been investigated. By stably transfecting a growth-regulatable murine β-cell line (βTC-Tet) with cFLIP, Cottet et al. demonstrated that cFLIP overexpression prevented cytokine-mediated apoptosis in vitro, although it did not preserve glucose-stimulated insulin release during this insult (24). Lentiviral mediated cFLIP overexpression in another murine β-cell line (NIT-1) has also been shown to prevent cytokine-mediated β-cell death in vitro (25). However, the in vitro benefit of cFLIP overexpression in islet β-cells has not been observed in vivo (26). This disappointing observation can most likely be attributed to the recent finding that cFLIP has a dual functionality that depends on expression levels and can under some circumstances actually promote apoptosis. Initially, it was determined that cFLIP deficient cells were more sensitive to death receptor-induced apoptosis, which is consistent with the role of cFLIP as a dominant-negative inhibitor of caspase-8 (23). However, recent data has shown that at high expression levels, cFLIP can activate procaspase-8 via heterodimerization (23). Since caspase-8 is also involved in cell cycle progression and proliferation, complete blockade of caspase-8 is undesirable, so it makes sense that at high levels of expression, cFLIP no longer inhibits its activation. In the context of islet transplantation, it is quite difficult if not impossible to consistently transduce all of the cells within an islet and establish uniform expression levels of a transgene using a viral vector (the most efficient way to deliver genes to intact islets). It is therefore not surprising that no in vivo experiments have been published that demonstrate a protective benefit of cFLIP overexpression in islet grafts. Also, considering that cFLIP only prevents the extrinsic pathway of apoptosis and as such has no effect on intrinsic signals such as hypoxia or ROS, it is unlikely that cFLIP alone could potently protect islet grafts.

In addition to transmitting apoptotic signals through receptor ligation, cytokine exposure can exert additional negative effects on islets by upregulating proteins including inducible nitric oxide synthase (iNOS) that lead to the generation of ROS within β-cells, activating the intrinsic pathway to apoptosis via the mitochondria. This secondary signal occurs via activation of the transcription factor Nuclear Factor κB, (NFκB). In healthy cells, NFκB is sequestered in the cytoplasm by the protein IκB kinase (IκK), and following inflammatory stimuli (i.e. cytokine or lipopolysaccaride (LPS) exposure, ROS generation following reperfusion injury), IκK is phosphorylated, which liberates NFκB and allows its translocation to the nucleus (27). Depending on the cell type and stimulus, NFκB can promote transcription of pro-apoptotic genes or anti-apoptotic genes. NFκB activation in β-cells has been associated with the upregulation of pro-inflammatory genes, including IL-1β, ICAM-1, and iNOS (27). An endogenous inhibitor of NFκB that prevents its activation has been identified, termed A20 (Figure 2-2). A20 is a zinc-ring finger protein not normally expressed in healthy cells and is instead upregulated by NFκB to provide feedback inhibition following its activation (28). The exact interaction between A20 and NFκB has been poorly understood until recently, when it was determined that A20 inhibits IκK phosphorylation indirectly by ubiquitin modification of the upstream kinases which act upon it, rendering them inactive (28). Since inhibition of NFκB with A20 could potentially inhibit extrinsic apoptotic signals as well as some intrinsic signals (i.e. reoxygenation injury), the protective benefit of its overexpression in islets has been studied. Adenovirally-mediated overexpresssion of A20 in rodent and human islets reduced cytokine-induced apoptosis and subsequent ROS production in vitro (29). A20 overexpression has also been associated with improved islet survival in vivo, using a syngeneic marginal islet mass transplantation model. Grey et al. showed that grafts containing 250 islets overexpressing A20 reversed diabetes in 75% of the recipients, compared to control grafts of 250 islets which only reversed diabetes in 20% of recipients (30). However, there was still evidence of caspase-3 in islet grafts from both cohorts, confirming that A20 does not inhibit all pathways of post-transplant apoptosis (30).

Despite its apparent promise, the long term prospects of A20 in clinical islet transplantation remain unclear. NFκB is not activated during hypoxia, a major determinant of post-transplant islet loss, so A20 overexpression cannot overcome this early trigger of β-cell death. It has also recently been determined that following oxidative stress, A20 actually enhanced ROS-induced apoptosis (31). Although NFκB activation is most often assumed to upregulate pro-inflammatory genes, a series of experiments by Fan et al. clearly demonstrated that the timing of NFκB activation significantly influences apoptotic outcomes following various stimuli (32). For example, inhibiting NFκB activation during reperfusion injury lead to increased apoptosis, while inhibiting NFκB activation prior to reperfusion injury prevented apoptosis (32). Thus, the timing of A20 expression may be critical in determining islet survival, which provides an additional layer of complexity to the clinical implementation of A20 therapy.

2.3.2 Inhibition of Intrinsic Signals

Given the mixed success of extrinsic apoptotic signal inhibition in islet transplantation, and the major contribution of intrinsic signals to islet death post-transplant (i.e. hypoxia, ROS formation during reperfusion, nutrient deprivation, etc.), strategies targeted at preventing mitochondria-mediated apoptosis have been extensively investigated. Many experiments have examined the impact of BCL-2 overexpression in β-cell lines and islets and have shown that it can inhibit apoptosis due to hypoxia, proinflammatory cytokines, staurosporine, serum withdrawal, and xenoreactive antibodies in vitro (33-36). However, these promising results have been met with mixed outcomes in animal models of transplantation. Adenovirally-mediated BCL-2 overexpression in macaque islets reduced the number of islets necessary to reverse diabetes in chemically diabetic nude mice, while βTC-Tet cells stably transfected with BCL-2 exhibited no survival advantage in vivo, despite enhanced proliferative capacity (26, 33, 36). Another anti-apoptotic BCL-2 family member, BCL-XL, has proven to be effective in preventing apoptosis triggered by IL-1β, staurosporine, and serum withdrawal in vitro (37). While transgenic overexpression of BCL-XL in islets prevented ROS-induced apoptosis in vitro, the transgenic animals were severely glucose intolerant due to a defect in mitochondrial nutrient metabolism and signaling for insulin secretion (38). Also, consistent with the inability of BCL-2 family members to inhibit extrinsic signals of apoptosis, transgenic expression of BCL-2 in islets had no effect on the T-cell mediated destruction of islets or diabetes onset in NOD mice (38). Thus, it remains unclear how much of an impact BCL-2 or BCL-XL overexpression might have in clinical islet transplantation.

2.3.3 Effector Caspase Inhibition to Prevent Both Extrinsic and Intrinsic Signals

Based on the studies cited above, it has become clear that inhibition of either the extrinsic or intrinsic pathway of apoptosis alone is insufficient to profoundly promote β-cell survival post-transplantation. Combining inhibitors of both pathways might enhance islet protection, but the difficulties in efficiently and reproducibly delivering and regulating multiple genes presents a significant challenge in islet transplantation. It would therefore be most attractive to inhibit apoptosis at a point beyond the convergence point of both pathways with a single gene product, in effect preventing all β-cell apoptosis, regardless of the specific stimuli. Over the past decade, a new family of apoptosis modulatory proteins has been identified, the inhibitors of apoptosis proteins (IAPs). These proteins share common structural motifs with unique functions that work in unison to block and possibly degrade multiple effector caspases late in the cascade of apoptotic triggers, thus preventing cell death at one common point for a variety of different activation pathways. Clinical observations have demonstrated that fluxes in endogenous cellular IAP protein levels have a lasting impact on a variety of human conditions, while new insights into the characteristics of the IAPs have suggested possible therapeutics for cancerous and immune-regulated diseases.

Although several non-IAP anti-apoptotic molecules have been identified, including cFLIP and the previously mentioned Bcl-2 family proteins, these proteins function at higher points in the apoptotic signaling sequence, limiting their effect to only one of the two major pathways. Conversely, the power of the IAPs comes from their ability to halt cell death late in the sequence of events, at the point where multiple apoptotic pathways come together, yet before any serious damage has occurred. The IAPs, originally identified in baculovirus as being involved in delaying host cell death during viral replication, have been found in organisms ranging from hydra to humans and include the human members human inhibitor of apoptosis proteins 1 and 2 (hIAP1/cIAP1 and hIAP2/cIAP2), X-linked inhibitor of apoptosis protein (XIAP), neuronal apoptosis inhibitory protein (NAIP), and survivin (reviewed in (39)). Each IAP functions in a similar fashion to prevent the proteolytic cleavage of a procaspase to its active form, most often blocking caspases 9, 7, and 3 (see 'XIAP' in Figure 2-2), but subtle structural differences account for the observed variation in potency between the IAPs.

As a family, the IAPs have been grouped according to two conserved structural features, the amino-terminus baculoviral inhibitor of apoptosis repeat (BIR) and carboxyl-terminus RING domains. Some IAPs have additional domains, including the caspase recruitment domain (CARD); however, these have not been linked to any specific functional characteristic nor are they necessary for apoptotic inhibition ((39)). Although not all of the IAPs possess a RING zinc finger domain (most notably survivin), all have a BIR region, and recent work has shown that the BIR domain is responsible and necessary for binding and hence preventing a procaspase from being cleaved to its active state (40). The function of the RING domain has not yet been as clearly defined, but it does promote autoubiquitination, most likely to aid in maintaining cellular homeostasis of IAP levels (41). Also, the RING domain might aid in stabilizing the IAP-procaspase interaction so that the BIR domain can bind more efficiently, or it may induce degradation of the procaspase via an ubiquitin pathway, although these hypotheses have not been confirmed.

Each of the five previously mentioned human IAP proteins has been examined for its individual anti-apoptotic activity. XIAP, HIAP1, and HIAP2 have three BIR domains (BIR1, BIR2, and BIR3) and one RING finger domain. Although the caspase inhibiting capacity varies between these proteins, they have all been show to directly inhibit the active forms of caspase-3 and caspase-7, both executioner caspases, by binding and blocking the enzyme active site with the BIR2 domain (39, 40). The BIR1 and BIR3 regions may assist in stabilizing protein-protein interactions, and XIAP studies demonstrate that BIR3 combined with the RING domain interacts with procaspase-9, preventing its activation by cytochrome c (40, 42). Only HIAP1 and HIAP2 have been show to suppress caspase-8 activation, which follows from the fact that these proteins were originally identified by their ability to bind a TNF-associated receptor (TRAF2), a death receptor that promotes apoptosis through caspase-8 activation (39). Survivin has a singular BIR domain and no RING finger motif; perhaps explaining why it is less effective compared to XIAP, HIAP1, or HIAP2 in antagonizing caspase-3 and caspase-7. Survivin is unique in that its expression has been tied to the cell cycle at the G_2/M transition and thus can only be detected in dividing cells, such as embryonic tissue (reviewed in (43)). NAIP works through a completely different mechanism, due mostly to its singular expression in neuronal tissues. Despite having three BIR domains, NAIP does not directly bind caspases; instead, it works through its BIR3 domain to specifically bind the neuron-restricted calcium-binding protein hippocalcin in the presence of calcium ions to synergistically downregulate caspase-3 and caspase-7 activity (44).

In addition to direct apoptosis inhibition, the IAPs can act through more complex, indirect mechanisms. In response to anti-apoptotic signals, NF-κB regulates the expression of stress response genes, including members of the IAP family. Interestingly, XIAP has been shown to stimulate this transcription factor, leading to an amplification of the anti-apoptotic response (42). Further studies involving XIAP have demonstrated that its functional protein levels can be upregulated through an internal ribosome entry site (IRES) on the 5' untranslated region of its mRNA during periods of cellular stress, even when transcription is generally halted (45, 46). Thus, when a cell is receiving stress signals, causing a downregulation of transcription and translation, the XIAP gene can be transcriptionally activated via NF-κB, while XIAP mRNA translation can continue due to its IRES motif. To further complicate the scenario, a novel endogenous inhibitor of IAPs has been discovered. The mitochondrial protein Smac/DIABLO is co-released with cytochrome c and binds the BIR domain(s) of IAPs, preventing the direct blockage of caspase activation (47, 48). In addition to its IAP inhibition, Smac/DIABLO helps in the formation of the apoptosome, further promoting cell death (49). These unique aspects of IAPs support the hypothesis that once a decision between survival and apoptosis has been taken, cellular mechanisms amplify the signaling pathways that substantiate this decision and make it irreversible.

The impact of IAP overexpression on β-cell survival following transplantation has yet to be examined, however, in other models of apoptotic disease, overexpression of XIAP, the most potent IAP, has produced impressive data. XIAP is known to prevent apoptosis triggered by a number of stimuli in vitro, including Fas ligation, cytokine exposure, hypoxia, reoxygenation injury, nutrient withdrawal, and chemotherapeutic agents (52, 54-56). Transient forebrain ischemia leads to apoptotic death of hippocampal neurons, causing a permanent defect in spatial learning, and ischemic apoptosis in the murine hippocampus was markedly reduced by overexpressing XIAP using an adenoviral vector that had been microinjected into the brain (54, 57). Also, XIAP overexpression has been shown to completely prevent retinal degeneration in an animal model of retinitis pigmentosa (58). Thus, XIAP represents a potential gene product that could provide a significant survival advantage to transplanted islets in the face of the diverse and plentiful apoptotic stimuli present following portal vein infusion and during the prolonged engraftment period.

2.4 Conclusions

Before clinical islet transplantation can be made accessible to a broader population of patients with T1DM, the current necessity for large amounts of donor islet tissue must be overcome. Since it has been determined that 60% or more of the islet tissue is lost to apoptosis soon after portal infusion, even in the absence of immune destruction, preventing this early post-transplant death would have an immediate impact on islet transplantation. The processes that negatively influence the survival of transplanted islets are diverse and complex, but they all ultimately lead to apoptosis. Significant efforts have been made to inhibit specific apoptotic triggers, either extrinsic (cFLIP, A20) or intrinsic (BCL-2, BCL-XL). These proteins have proven quite effective in enhancing β-cell survival in vitro, following exposure to the particular stimulus each protein is known to block. However, reproducing the protective effect using transplanted islets has been difficult and largely unfruitful. The best strategy to significantly enhance islet survival post-transplantation may be to overexpress IAP proteins (i.e. XIAP), which potently inhibit effector caspases that function late in apoptosis, effectively preventing cell death triggered by extrinsic and intrinsic pathways at the same time. If IAP overexpression did prevent the widespread loss of transplanted islets in the early engraftment period, it would reduce the amount of tissue required to restore euglycemia, and in this regard broaden the availability of cadaveric islet transplantation and possibly open the door to living-donor islet transplantation. Also, the islets would presumably be more robust and functional early on, which may contribute to stable, prolonged graft function and insulin independence.

Finally, much less apoptotic donor antigens would be released into the recipient, which could reduce the requirement for immunosuppressive drug therapy. If these outcomes could be achieved, islet transplantation would be a therapeutic option for most patients with T1DM, especially children.

The studies presented in this thesis test the hypothesis that high levels of XIAP expression in islets will enhance β-cell survival during periods of hypoxia and reoxygenation, and thus reduce the islet mass necessary to restore euglycemia in diabetic animals. This hypothesis was tested in a murine β-cell line (βTC-Tet) (chapter 3), human islets (chapter 4), and neonatal porcine islets (chapter 5).

2.5 References

1. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000;343(4):230-8.

2. Slezak LA, Andersen DK. Pancreatic resection: effects on glucose metabolism. World J Surg 2001;25(4):452-60.

3. Weir GC, Bonner-Weir S. Five stages of evolving beta-cell dysfunction during progression to diabetes. Diabetes 2004;53 Suppl 3:S16-21.

4. Ryan EA, Lakey JR, Paty BW, Imes S, Korbutt GS, Kneteman NM, et al. Successful islet transplantation: continued insulin reserve provides long-term glycemic control. Diabetes 2002;51(7):2148-57.

5. Biarnes M, Montolio M, Nacher V, Raurell M, Soler J, Montanya E. Beta-cell death and mass in syngeneically transplanted islets exposed to short- and long-term hyperglycemia. Diabetes 2002;51(1):66-72.

6. Davalli AM, Ogawa Y, Ricordi C, Scharp DW, Bonner-Weir S, Weir GC. A selective decrease in the beta cell mass of human islets transplanted into diabetic nude mice. Transplantation 1995;59(6):817-20.

7. Carlsson PO, Palm F, Andersson A, Liss P. Markedly decreased oxygen tension in transplanted rat pancreatic islets irrespective of the implantation site. Diabetes 2001;50(3):489-95.

8. Carlsson PO, Palm F, Mattsson G. Low revascularization of experimentally transplanted human pancreatic islets. J Clin Endocrinol Metab 2002;87(12):5418-23.

9. Dionne KE, Colton CK, Yarmush ML. Effect of hypoxia on insulin secretion by isolated rat and canine islets of Langerhans. Diabetes 1993;42(1):12-21.

10. Giuliani M, Moritz W, Bodmer E, Dindo D, Kugelmeier P, Lehmann R, et al. Central necrosis in isolated hypoxic human pancreatic islets: evidence for postisolation ischemia. Cell Transplant 2005;14(1):67-76.

11. Moberg L, Johansson H, Lukinius A, Berne C, Foss A, Kallen R, et al. Production of tissue factor by pancreatic islet cells as a trigger of detrimental thrombotic reactions in clinical islet transplantation. Lancet 2002;360(9350):2039-45.

12. Ozmen L, Ekdahl KN, Elgue G, Larsson R, Korsgren O, Nilsson B. Inhibition of thrombin abrogates the instant blood-mediated inflammatory reaction triggered by isolated human islets: possible application of the thrombin inhibitor melagatran in clinical islet transplantation. Diabetes 2002;51(6):1779-84.

13. Paraskevas S, Maysinger D, Wang R, Duguid TP, Rosenberg L. Cell loss in isolated human islets occurs by apoptosis. Pancreas 2000;20(3):270-6.

14. Davalli AM, Scaglia L, Zangen DH, Hollister J, Bonner-Weir S, Weir GC. Vulnerability of islets in the immediate posttransplantation period. Dynamic changes in structure and function. Diabetes 1996;45(9):1161-7.

15. Moritz W, Meier F, Stroka DM, Giuliani M, Kugelmeier P, Nett PC, et al. Apoptosis in hypoxic human pancreatic islets correlates with HIF-1alpha expression. Faseb J 2002;16(7):745-7.

16. Hengartner MO. The biochemistry of apoptosis. Nature 2000;407(6805):770-6.

17. Boatright KM, Salvesen GS. Mechanisms of caspase activation. Curr Opin Cell Biol 2003;15(6):725-31.

18. Chan SL, Yu VC. Proteins of the bcl-2 family in apoptosis signalling: from mechanistic insights to therapeutic opportunities. Clin Exp Pharmacol Physiol 2004;31(3):119-28.

19. Green DR. Apoptotic pathways: paper wraps stone blunts scissors. Cell 2000;102(1):1-4.

20. Hui H, Dotta F, Di Mario U, Perfetti R. Role of caspases in the regulation of apoptotic pancreatic islet beta-cells death. J Cell Physiol 2004;200(2):177-200.

21. Suarez-Pinzon W, Sorensen O, Bleackley RC, Elliott JF, Rajotte RV, Rabinovitch A. Beta-cell destruction in NOD mice correlates with Fas (CD95) expression on beta-cells and proinflammatory cytokine expression in islets. Diabetes 1999;48(1):21-8.

22. Loweth AC, Williams GT, James RF, Scarpello JH, Morgan NG. Human islets of Langerhans express Fas ligand and undergo apoptosis in response to interleukin-1beta and Fas ligation. Diabetes 1998;47(5):727-32.

23. Peter ME. The flip side of FLIP. Biochem J 2004;382(Pt 2):e1-3.

24. Cottet S, Dupraz P, Hamburger F, Dolci W, Jaquet M, Thorens B. cFLIP protein prevents tumor necrosis factor-alpha-mediated induction of caspase-8-dependent apoptosis in insulin-secreting betaTc-Tet cells. Diabetes 2002;51(6):1805-14.

25. Fenjves ES, Ochoa MS, Gay-Rabinstein C, Ricordi C, Curran MA. Retrovirally transferred genes inhibit apoptosis in an insulin-secreting cell line: implications for islet transplantation. Cell Transplant 2004;13(5):489-96.

26. Cattan P, Rottembourg D, Cottet S, Tardivel I, Dupraz P, Thorens B, et al. Destruction of conditional insulinoma cell lines in NOD mice: a role for autoimmunity. Diabetologia 2003;46(4):504-10.

27. Ho E, Bray TM. Antioxidants, NFkappaB activation, and diabetogenesis. Proc Soc Exp Biol Med 1999;222(3):205-13.

28. Heyninck K, Beyaert R. A20 inhibits NF-kappaB activation by dual ubiquitin-editing functions. Trends Biochem Sci 2005;30(1):1-4.

29. Grey ST, Arvelo MB, Hasenkamp W, Bach FH, Ferran C. A20 inhibits cytokine-induced apoptosis and nuclear factor kappaB-dependent gene activation in islets. J Exp Med 1999;190(8):1135-46.

30. Grey ST, Longo C, Shukri T, Patel VI, Csizmadia E, Daniel S, et al. Genetic engineering of a suboptimal islet graft with A20 preserves beta cell mass and function. J Immunol 2003;170(12):6250-6.

31. Storz P, Doppler H, Ferran C, Grey ST, Toker A. Functional dichotomy of A20 in apoptotic and necrotic cell death. Biochem J 2005;387(Pt 1):47-55.

32. Fan C, Yang J, Engelhardt JF. Temporal pattern of NFkappaB activation influences apoptotic cell fate in a stimuli-dependent fashion. J Cell Sci 2002;115(Pt 24):4843-53.

33. Dupraz P, Rinsch C, Pralong WF, Rolland E, Zufferey R, Trono D, et al. Lentivirus-mediated Bcl-2 expression in betaTC-tet cells improves resistance to hypoxia and cytokine-induced apoptosis while preserving in vitro and in vivo control of insulin secretion. Gene Ther 1999;6(6):1160-9.

34. Allison J, Thomas H, Beck D, Brady JL, Lew AM, Elefanty A, et al. Transgenic overexpression of human Bcl-2 in islet beta cells inhibits apoptosis but does not prevent autoimmune destruction. Int Immunol 2000;12(1):9-17.

35. Contreras JL, Bilbao G, Smyth C, Eckhoff DE, Xiang XL, Jenkins S, et al. Gene transfer of the Bcl-2 gene confers cytoprotection to isolated adult porcine pancreatic islets exposed to xenoreactive antibodies and complement. Surgery 2001;130(2):166-74.

36. Contreras JL, Bilbao G, Smyth CA, Jiang XL, Eckhoff DE, Jenkins SM, et al. Cytoprotection of pancreatic islets before and soon after transplantation by gene transfer of the anti-apoptotic Bcl-2 gene. Transplantation 2001;71(8):1015-23.

37. Klein D, Ribeiro MM, Mendoza V, Jayaraman S, Kenyon NS, Pileggi A, et al. Delivery of Bcl-XL or its BH4 domain by protein transduction inhibits apoptosis in human islets. Biochem Biophys Res Commun 2004;323(2):473-8.

38. Zhou YP, Pena JC, Roe MW, Mittal A, Levisetti M, Baldwin AC, et al. Overexpression of Bcl-x(L) in beta-cells prevents cell death but impairs mitochondrial signal for insulin secretion. Am J Physiol Endocrinol Metab 2000;278(2):E340-51.

39. LaCasse EC, Baird S, Korneluk RG, MacKenzie AE. The inhibitors of apoptosis (IAPs) and their emerging role in cancer. Oncogene 1998;17(25):3247-59.

40. Sun C, Cai M, Meadows RP, Xu N, Gunasekera AH, Herrmann J, et al. NMR structure and mutagenesis of the third Bir domain of the inhibitor of apoptosis protein XIAP. J Biol Chem 2000;275(43):33777-81.

41. Yang Y, Fang S, Jensen JP, Weissman AM, Ashwell JD. Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. Science 2000;288(5467):874-7.

42. Hofer-Warbinek R, Schmid JA, Stehlik C, Binder BR, Lipp J, de Martin R. Activation of NF-kappa B by XIAP, the X chromosome-linked inhibitor of apoptosis, in endothelial cells involves TAK1. J Biol Chem 2000;275(29):22064-8.

43. Reed JC, Bischoff JR. BIRinging chromosomes through cell division--and survivin' the experience. Cell 2000;102(5):545-8.

44. Mercer EA, Korhonen L, Skoglosa Y, Olsson PA, Kukkonen JP, Lindholm D. NAIP interacts with hippocalcin and protects neurons against calcium-induced cell death through caspase-3-dependent and -independent pathways. Embo J 2000;19(14):3597-607.

45. Holcik M, Lefebvre C, Yeh C, Chow T, Korneluk RG. A new internal-ribosome-entry-site motif potentiates XIAP-mediated cytoprotection. Nat Cell Biol 1999;1(3):190-2.

46. Holcik M, Yeh C, Korneluk RG, Chow T. Translational upregulation of X-linked inhibitor of apoptosis (XIAP) increases resistance to radiation induced cell death. Oncogene 2000;19(36):4174-7.

47. Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, et al. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell 2000;102(1):43-53.

48. Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell 2000;102(1):33-42.

49. Srinivasula SM, Datta P, Fan XJ, Fernandes-Alnemri T, Huang Z, Alnemri ES. Molecular determinants of the caspase-promoting activity of Smac/DIABLO and its role in the death receptor pathway. J Biol Chem 2000;275(46):36152-7.

50. Datta R, Oki E, Endo K, Biedermann V, Ren J, Kufe D. XIAP regulates DNA damage-induced apoptosis downstream of caspase-9 cleavage. J Biol Chem 2000;275(41):31733-8.

51. Fong WG, Liston P, Rajcan-Separovic E, St Jean M, Craig C, Korneluk RG. Expression and genetic analysis of XIAP-associated factor 1 (XAF1) in cancer cell lines. Genomics 2000;70(1):113-22.

52. Straszewski-Chavez SL, Abrahams VM, Funai EF, Mor G. X-linked inhibitor of apoptosis (XIAP) confers human trophoblast cell resistance to Fas-mediated apoptosis. Mol Hum Reprod 2004;10(1):33-41.

53. Tamm I, Kornblau SM, Segall H, Krajewski S, Welsh K, Kitada S, et al. Expression and prognostic significance of IAP-family genes in human cancers and myeloid leukemias. Clin Cancer Res 2000;6(5):1796-803.

54. Xu D, Bureau Y, McIntyre DC, Nicholson DW, Liston P, Zhu Y, et al. Attenuation of ischemia-induced cellular and behavioral deficits by X chromosome-linked inhibitor of apoptosis protein overexpression in the rat hippocampus. J Neurosci 1999;19(12):5026-33.

55. Schimmer AD. Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice. Cancer Res 2004;64(20):7183-90.

56. Simons M, Beinroth S, Gleichmann M, Liston P, Korneluk RG, MacKenzie AE, et al. Adenovirus-mediated gene transfer of inhibitors of apoptosis protein delays apoptosis in cerebellar granule neurons. J Neurochem 1999;72(1):292-301.

57. Xu DG, Crocker SJ, Doucet JP, St-Jean M, Tamai K, Hakim AM, et al. Elevation of neuronal expression of NAIP reduces ischemic damage in the rat hippocampus. Nat Med 1997;3(9):997-1004.

58. Petrin D, Baker A, Coupland SG, Liston P, Narang M, Damji K, et al. Structural and functional protection of photoreceptors from MNU-induced retinal

degeneration by the X-linked inhibitor of apoptosis. Invest Ophthalmol Vis Sci 2003;44(6):2757-63.

59. Kasof GM, Gomes BC. Livin, a novel inhibitor of apoptosis protein family member. J Biol Chem 2001;276(5):3238-46.

Chapter 3

XIAP Overexpression in Islet β-cells Enhances Engraftment and Minimizes Hypoxia-Reperfusion Injury

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3.1 Introduction

T1DM is a chronic, progressive autoimmune disorder that results in the destruction of the insulin-producing β-cells in the islets of Langerhans within the pancreas. Until recently, insulin injections were the only treatment option available to the nearly 200 million patients with diabetes worldwide, but despite concerted efforts to regulate blood glucose levels, chronic exposure to hyperglycemia often leads to secondary complications including nephropathy and retinopathy. The recently introduced Edmonton protocol can provide excellent glycemic control and insulin independence in a population of patients, but the procedure requires >10,000 IE/kg, generally derived from two or more donors (1). Also, recipients must take life-long immunosuppression, and this limits the procedure to a highly select group of patients with the severest forms of diabetes. The current method of human islet isolation and purification causes hypoxic stress to which β-cells are extremely sensitive, and similarly the microenvironment immediately after portal infusion negatively influences islet graft survival (2). A substantial portion of the transplanted tissue becomes nonfunctional very soon after injection for a number of causes, including immune response to tissue factor expression in islets and hypoxia-induced damage leading to islet apoptosis (3, 4).

The islet microenvironment within a healthy pancreas facilitates β-cell function by maximizing interaction between the β-cells and circulating blood. Consequently, β-cells survive optimally when they are exposed to highly oxygenated blood (pO_2 of 40 mmHg) in nutrient rich surroundings (5). Regardless of the transplantation site, the ischemic period immediately after transplantation leads to hypoxia (pO_2 of 5-10 mmHg or approximately <1% O_2), and upon reperfusion the islets are exposed to severe oxidative stress (5). Consequently, a significant fraction of the islet graft is lost due to apoptosis, which begins within hours post-transplant, peaks at day 2-3, and decreases subsequently until day 14, when what remains of the graft has stabilized (6, 7). During the period of graft stabilization, the islet tissue still remains susceptible to enhanced immunological attack (8).

For the reasons listed above it has been estimated that up to 60% of transplanted β-cell mass is lost to apoptosis even in syngeneic situations, implying that interventional strategies with anti-apoptotic agents may be crucial for islet mass preservation in the transplant setting (9). Indeed, in clinical islet transplant grafts, it has been estimated that more than two thirds of the implanted mass fails to engraft (10). The impact of anti-apoptotic genes in the acute post-transplant period remains unknown. The mitochondrial apoptotic pathway inhibitors Bcl-2 and Bcl-XL have been shown to prevent β-cell death from hypoxia and/or proinflammatory cytokines in vitro, but have failed to prevent allograft rejection in vivo (11-13). A20, which inhibits NF-κB activation, has been shown to protect β-cells from cytokine- and Fas-mediated killing in vitro and to reduce the β-cell mass required for syngeneic islet transplantation (14, 15).

Although studies using IAP family proteins have shown promise in other rodent models of apoptotic disease, the impact of IAP overexpression on β-cell survival remains to be examined (16-18). The most potent IAP family member is

XIAP, which prevents the activation of caspases 3, 7 and 9, effector caspases that function late in apoptosis beyond the convergence point of most apoptotic pathways. These include extracellular signals, such as Fas-Fas Ligand interactions, and intracellular signals, such as DNA damage or oxidative stress. In rat stroke models, significantly improved recoveries have been obtained when the target neural tissue has been engineered to resist apoptosis by adenoviral mediated over-expression of XIAP, and the same should be possible for pancreatic β-cells, which share similar sensitivity with neurons to oxidative stress (19).

In the present study, XIAP protein was overexpressed using an adenoviral vector in the highly differentiated, glucose responsive murine β-cell line, βTC-Tet. This cell line was generated from transgenic mice and possesses the unique capacity for tetracycline-regulated growth arrest, which allows its use in vivo without the inevitable hypoglycemic complications observed with the use of other β-cell line transplants (20, 21). This particular β-cell line is ideal for examining potentially protective genes in the context of islet transplantation because it can be easily transduced with high efficiency and remains glucose sensitive during periods of proliferation as well as during growth arrest (20). The aim of this study was to assess the protective effects of XIAP overexpression during hypoxia-reperfusion injury and cytokine-induced stress in vitro, and to determine the impact of XIAP-mediated apoptosis inhibition in β-cells during the immediate post-transplant period in vivo using NOD-RAG1$^{-/-}$ recipients.

3.2 Materials and Methods

3.2.1 Cell culture

βTc-Tet cells are an islet β-cell line derived from the insulinomas of C3H transgenic mice expressing tetracycline-regulated SV40 TAg (20, 21). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 450 mg/dL glucose, 1 mM Na-pyruvate, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen Canada, Burlington, Ontario) in 5% CO_2 at 37 °C. In all in vitro studies, the cells were allowed to continuously proliferate.

3.2.2 Adenoviral stocks and transduction

Adenoviral constructs were prepared as previously described by Liston et al. (16). Ad-LacZ and Ad-XIAP virus stocks with identical adenoviral backbones were titred using standard plaque assays on 293A cells, and then stored as frozen aliquots. Subconfluent monolayers of βTc-Tet were transduced at 10 virus particles (V.P.)/cell with Ad-XIAP or Ad-LACZ for 1h in a minimal volume of DMEM culture medium (i.e. 2.0 mL in a 75 cm^2 dish) and incubated at 37 °C in 5% CO_2. Following transduction, the cells were washed twice with culture medium and incubated overnight (transplant studies) or 48h prior to all in vitro assays.

3.2.3 Analysis of adenoviral transgene expression

Ad-XIAP expression-XIAP protein was detected using anti-XIAP monoclonal antibody (Clone 48, BD Pharmingen, Mississauga, ON). Lysates were prepared from $5x10^5$ cells (or 500 islets) 48h following Ad-XIAP transduction at 10 V.P./cell

(10^4/islet) in EDTA-free protease inhibitor cocktail (Roche) supplemented with 0.1% SDS, followed by bicinchoninic acid (BCA) assays to determine protein concentration (Pierce Chemical Co., Rockford, IL). Western blot analysis was performed by running 15 μg total protein extract on a 10% SDS-PAGE gel, followed by transfer to nitrocellulose (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked for 1h in PBS containing 0.05% Tween-20 (PBS-T) and 5% dry skim milk (blocking buffer), and then incubated for 1h with a 1:250 dilution of anti-XIAP in blocking buffer. After washing the membranes in PBS-T, they were incubated with peroxidase-labeled goat anti-mouse IgG (Amersham Pharmacia Biotech) at a 1:2500 dilution in blocking buffer for 1h. The ECL detection system (Amersham Pharmacia Biotech) was used with BioMax-MR film (Kodak) to visualize XIAP protein bands. *Ad-LACZ expression*-βGalactosidase activity was assayed using the technique developed by Sanes et al. (22).

3.2.4 Hypoxia Studies

Ad-XIAP or Ad-LacZ transduced βTC-Tet cells were cultured in <1% oxygen conditions using a system developed by R.T. Kilani et al. (23). Briefly, 12-well plates containing 5×10^5 Ad-XIAP or Ad-LacZ transduced βTc-Tet cells were placed into a modular-incubator chamber (Billups-Rothenberg, DeMar, CA) at 37 °C. Surrounding air within the chamber was replaced with nitrogen containing 5% CO_2 by a controlled purge for 15 min. at 200 mL/sec. The oxygen content within this system is approximately 10 mmHg (≈1% O_2) (23). Following 24h within the chamber, the plates were removed and either analyzed immediately or cultured for an additional 24h under normal conditions (20% O_2, 5% CO_2, 37 °C) to induce re-oxygenation injury. Cells cultured in either hypoxic or hypoxic/reoxygenation environments were compared to control cells cultured for the same time period under normal conditions.

3.2.5 Cytokine Treatment

Samples containing $5x10^5$ untreated, Ad-LACZ, or Ad-XIAP transduced βTC-Tet cells were grown on chamber slides (4 chambers/slide, Lab-Tek, Fisher) and exposed to the murine cytokines IL-1β (100 U/mL, Cell Signaling Technologies, Beverly, MA), TNFα (1000 U/mL, Peprotech, Rocky Hill, NJ), and IFNγ (1000 U/mL, Peprotech) in DMEM culture medium for 72h before being analyzed.

3.2.6 Apoptosis assays

The number of apoptotic β-cells present following exposure to hypoxia, hypoxia with reoxygenation, or cytokine treatment was determined using the complementary techniques of Annexin V and TUNEL staining. *Annexin V-* Freshly dispersed βTC-Tet cells were labeled with Annexin V-FITC (Oncogene Research, San Diego, CA). *TUNEL-* Dispersed βTC-Tet cells were fixed in 4% paraformaldehyde and apoptotic nuclei were labeled with FITC-dUTP (Roche) using the TdT enzyme. DAPI (4',6-diamino-2-phenylindol, Molecular Probes, Eugene, OR) nuclear counterstaining was used to detect all cells present in the sample. For both methods, cells were stained in suspension, applied directly to slides with coverslips, and analyzed immediately using fluorescent microscopy (UV excitation at 240-380 nm). Each sample was stained in triplicate, and at least 500 cells were counted per slide. All experimental samples were compared to non-treated control cultures to determine parallel rates of spontaneous apoptosis.

3.2.7 Cell viability assays

Cell viability was measured with the colorimetric assay that detects the reduction of 3-(4,5-dimethylthiazolyl-2) 2,5-diphenyltetrazolium bromide (MTT, Sigma) into a blue formazan product in metabolically active cells (24).

3.2.8 Glucose-Stimulated Insulin Release Assays

For each sample, duplicate aliquots containing 5×10^5 cells were incubated in low-glucose medium (DMEM containing 100 mg/dL D-Glucose supplemented with 10% FCS) for 10 min. The media was then removed and replaced with either low glucose medium or high glucose medium (DMEM containing 450 mg/dL D-Glucose supplemented with 10% FCS) and incubated for 2h at 37 °C, 5% CO_2. Aliquots of 250 μL supernatant from each sample were analyzed in triplicate for rodent insulin content using a radioimmunoassay (Linco Research, St. Charles, MO). The fold stimulation for each experimental condition was calculated by dividing the mean insulin released from cells cultured in high glucose medium by the mean insulin released from cells cultured in parallel in low glucose medium.

3.2.9 Animals

NOD-RAG$^{-/-}$ mice (NOD.129S7(B6)-*Rag1*^{tm1Mom}/J), which are immunodeficient due to a lack of mature T or B cells, were obtained from Jackson Labs and housed under specific pathogen-free conditions. All mice were cared for according to the guidelines of the Canadian Council on Animal Care and ethical approval was obtained from the animal welfare committee at the University of Alberta.

3.2.10 β-cell transplantation studies

Diabetes was induced chemically using Streptozotocin (STZ, 200 mg/kg (I.P.), Sigma) in male NOD-RAG$^{-/-}$ mice. Animals were considered to be diabetic after two consecutive blood glucose measurements ≥325 mg/dL. To transplant βTC-Tet cells subcutaneously, the injection site on the back was shaved with a sterile scalpel blade (≈1 x 1 cm area), the skin raised up between thumb and forefinger, and a graft containing 4×10^6 cells (either Ad-LacZ or Ad-XIAP transduced) was injected in ≤200 μl volume using a 23g needle and insulin syringe. Immediately after withdrawing the needle, a spot of 'Liquid Bandage' (Band-Aid Brand, Johnson and Johnson, New Brunswick, New Jersey) was used to seal the hole. This blocked any graft material from leaking out and prevented infection at the injection site.

In all transplantation studies, the blood glucose of each animal was measured every day for the first 7 days and on alternating days thereafter using a One-Touch Ultra Glucometer (Johnson and Johnson). The transplant was considered successful when the nonfasting blood glucose returned to normal (<220 mg/dL).

3.2.11 Glucose Tolerance Tests

Wild-type NOD-RAG$^{-/-}$ mice or those transplanted with XIAP-expressing or βGAL-expressing grafts that had been stabilized for at least 30 days were fasted overnight and injected I.P. with prewarmed 50% dextrose in Ringer's solution at 2 g/kg body weight. The animals were monitored for blood glucose levels at 0, 15, 30, 60, 120, and 180 minutes following injection.

3.2.12 Statistical Methods

All statistical analysis was carried out using SigmaPlot 2000 (SPSS, Inc. Chicago, IL, USA). Results are expressed as mean ± SEM. Student's *t*-tests for paired and unpaired data were used to compare results from in vitro hypoxia, hypoxia with reoxygenation, and cytokine-treated β-cell experiments.

3.3 Results

3.3.1 Expression of Ad-XIAP and Ad-LACZ in βTC-Tet cells

To study the effect on β-cell survival, a first generation recombinant adenovirus containing the human XIAP cDNA driven by the chicken β-actin promoter (Ad-XIAP) was used to transduce βTC-Tet cells. Western blot analysis for XIAP revealed that following transduction with Ad-XIAP both βTC-Tet cells and purified islets from NOD-RAG knock-out mice expressed high levels of the recombinant human protein (Fig.3-1A). No antibody currently exists that detects only human XIAP--all cross-react with mouse XIAP (cross-reactive mouse XIAP appears as a faint band at 57 KDa in the exposure shown in Fig. 3-1A), so we were not able to quantify the number of hXIAP-expressing βTC-Tet cells or NOD-RAG islet cells by immunohistochemistry. However, when a similarly grown and titred Ad-LACZ virus (a first generation recombinant adenovirus containing β-galactosidase driven by the chicken β-actin promoter) was used to transduce βTC-Tet cells at the same multiplicity of infection (MOI) as used for the Ad-XIAP in Fig. 3-1A, X-GAL staining showed that 98.1±1.3% of βTC-Tet cells were expressing βGal (Fig. 3-1C). This compares to complete absence of βGal staining for untransduced βTC-Tet cells (Fig. 3-1B). Ad-XIAP or Ad-LACZ transduced βTC-Tet cells showed no loss in their ability to secrete insulin in response to glucose stimulation (Fig. 3-3, untreated controls). Also, Ad-XIAP transduced βTC-Tet cells did not exhibit differences in proliferation rates compared to untransduced or Ad-LACZ transduced cells. This is in contrast to a previous report where βTC-Tet cells stably transfected with the anti-apoptotic gene Bcl-2 exhibited an increase in proliferation rate (11).

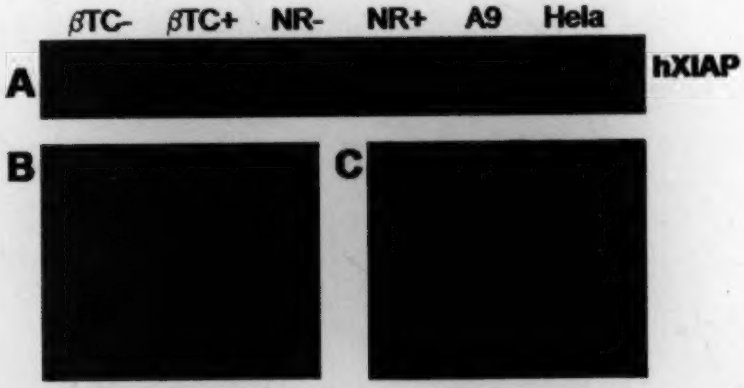

Figure 3-1: Adenoviral-mediated expression of Hu-XIAP or βGalactosidase in βTC-Tet cells and NOD-RAG$^{-/-}$ islets.

(A) βTC-Tet cell lysates prepared from untransduced (βTC-) or Ad-XIAP transduced (βTC+) cells were run alongside similar lysates made from untransduced NOD-RAG$^{-/-}$ (NR-) or Ad-XIAP transduced (NR+) islets. Transductions utilized MOI's of 10 V.P. per βTC-Tet cell and 10^{4} V.P. per islet; cells were harvested 48h after transduction, and 15 μg of lysate was run per lane. The Western blot was probed with an anti-Hu-XIAP monoclonal antibody and human XIAP appears in the transduced cells as a band of the expected size (57 kDa). Lane A9 contains a lystate made from untransduced murine A9 cells (10 μg). As a positive control, the lane labeled 'Hela' contains 15 μg of untransfected Hela cell lysate; this cell line is known to express high levels of endogenous Hu-XIAP. The lower two frames show untransduced (B) or Ad-LACZ transduced (C) βTC-Tet cells (MOI 10 V.P. per cell) stained with X-GAL after 48h in culture. 98.1±1.3% of the cells in the Ad-LACZ transduced flask stained positive for β-Gal. Similar results were obtained in three independent experiments. Cells are shown at 100x magnification.

3.3.2 Apoptosis due to hypoxia and hypoxia-reoxygenation insult is inhibited in the presence of XIAP

The ability of XIAP to prevent apoptosis during periods of hypoxia and following hypoxia-reperfusion type insult was examined in vitro using a hypoxic chamber apparatus. Following exposure to hypoxia for 24h ($<1\%$ O_2, 5% CO_2), 50% of the LACZ transduced βTC-Tet cells died (MTT assay, Fig. 3-2A), and this was reflected by increased numbers of apoptotic cells in the LACZ group (assayed by Annexin V staining and TUNEL, Figs. 3-2B and C).

In contrast the XIAP transduced cells survived much better and showed considerably lower levels of apoptosis. This effect of XIAP was further magnified when cells were subjected to 24h of hypoxia followed by 24h of culture in normal air (this mimics hypoxia-reperfusion injury); levels of cell viability and rates of apoptosis in XIAP transduced cultures returned to levels quite near those of control, untreated cultures. Although XIAP overexpression protected β-cells from hypoxia-induced apoptosis and enhanced β-cell viability, glucose-stimulated insulin secretion after 24h of hypoxia was considerably compromised (Fig. 3-3A). However, XIAP overexpression allowed the β-cells to functionally recover over time following hypoxia-reoxygenation (i.e. XIAP transduced cells showed improved insulin secretion over 72h, demonstrating evidence of partial recovery, while LACZ controls completely lost their ability to secrete insulin following the reoxygenation insult (Fig. 3-3A)).

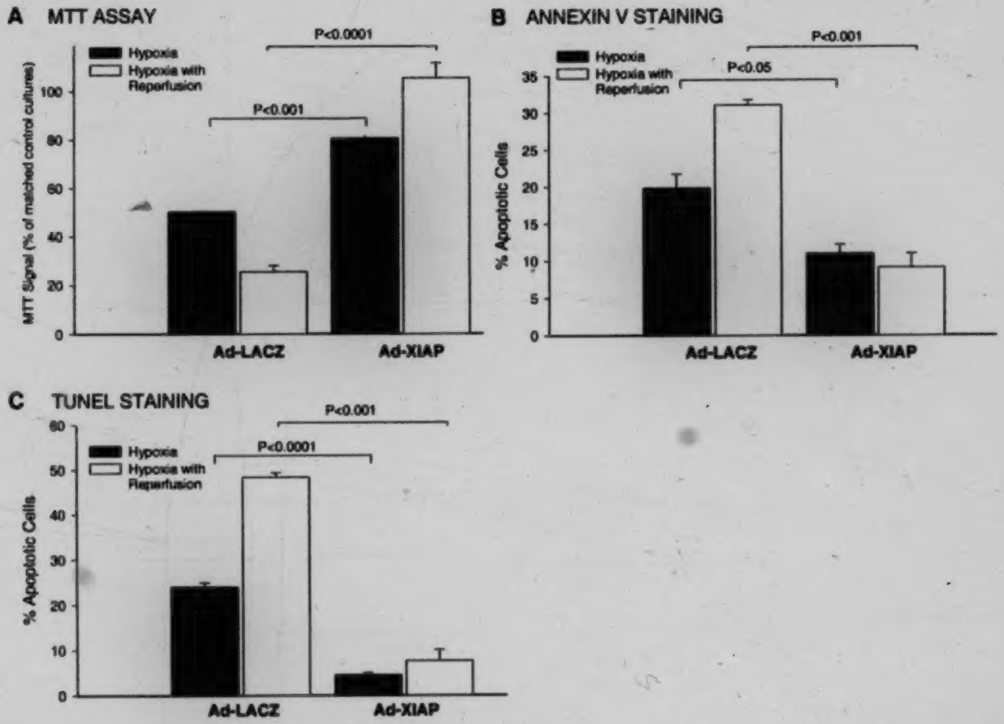

Figure 3-2: Hu-XIAP overexpression protects transduced βTC-Tet cells from hypoxia and hypoxia-'reperfusion' injury (i.e. re-oxygenation), as demonstrated by improved cell viability (MTT Assay, A) and decreased numbers of apoptotic cells (Annexin V Staining, B and TUNEL Staining C).

Hypoxia was achieved in Ad-LACZ and Ad-XIAP transduced βTC-Tet cells by incubating flasks in <1% O_2, 5% CO_2 for 24h. Hypoxia with re-oxygenation was accomplished when βTC-Tet cell cultures maintained for 24h in hypoxic conditions were transferred back to normal conditions (20% O_2, 5% CO_2) for a further 24h. Untransduced βTC-Tet cells were also exposed to hypoxia and reoxygenation during these experiments, and they demonstrated similar levels of apoptosis as LacZ-transduced cells (no statistical difference, see Figure 5-1, p.123). Values in (A) are expressed as a % of matched control cultures, which were not subjected to hypoxia, but simply left in a standard tissue culture incubator. The data are presented as mean±SE and are representative of two independent experiments, each performed in triplicate.

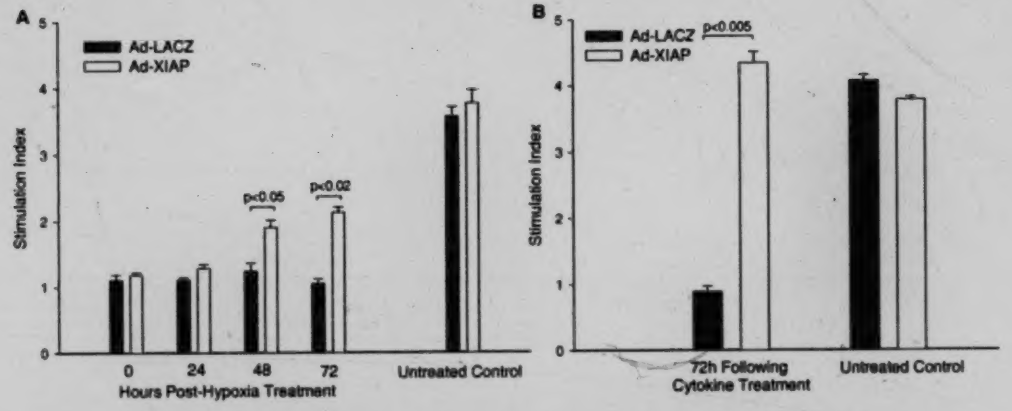

Figure 3-3: XIAP overexpression protects transduced βTC-Tet cells from hypoxia and hypoxia-'reperfusion' injury-induced and cytokine treatment-induced loss of function.

(A) Hypoxia was achieved in Ad-LACZ and Ad-XIAP transduced βTC-Tet cells by incubating flasks in <1% O_2, 5% CO_2 for 24h. When βTC-Tet cell cultures maintained for 24h in hypoxic conditions were transferred back to normal conditions ("reperfusion," 20% O_2, 5% CO_2), glucose-stimulated insulin release assays were performed at time 0, 24, 48, and 72h. (B) A cocktail containing pro-inflammatory cytokines [IL-1β (Cf 10^2 U/mL), TNFα (Cf 10^3 U/mL), and IFNγ (Cf 10^3 U/mL)] was added to βTC-Tet cells that had been transduced with Ad-XIAP or Ad-LacZ 48h prior. Following 72h of cytokine exposure, glucose-stimulated insulin release assays were performed. Insulin levels were analyzed in triplicate, and values are given as mean ±SE. Data are representative of three independent experiments, with each sample assayed in triplicate.

3.3.3 Cytokine-induced apoptosis is inhibited by XIAP overexpression

An important factor affecting the survival of β-cell grafts in the early post-transplant period is the local secretion of proinflammatory cytokines including IL-1β, TNFα, and IFNγ by resident macrophages, dendritic cells, and NK cells. To evaluate whether XIAP overexpression could prevent cytokine-mediated apoptosis, XIAP or LACZ transduced βTC-Tet cells were treated with a cocktail of murine cytokines as outlined in Fig. 3-4. Cell viability was well-preserved in β-cells expressing XIAP (MTT assay, Fig. 3-4A), which is supported by the lower numbers of Annexin V or TUNEL positive apoptotic cells in the XIAP transduced samples (Figs. 3-4B and 3-4C). XIAP overexpression also prevented the loss of glucose-stimulated insulin secretion following cytokine treatment, while cytokine treated Ad-LACZ transduced cells completely lost their glucose responsiveness (Fig. 3-3B).

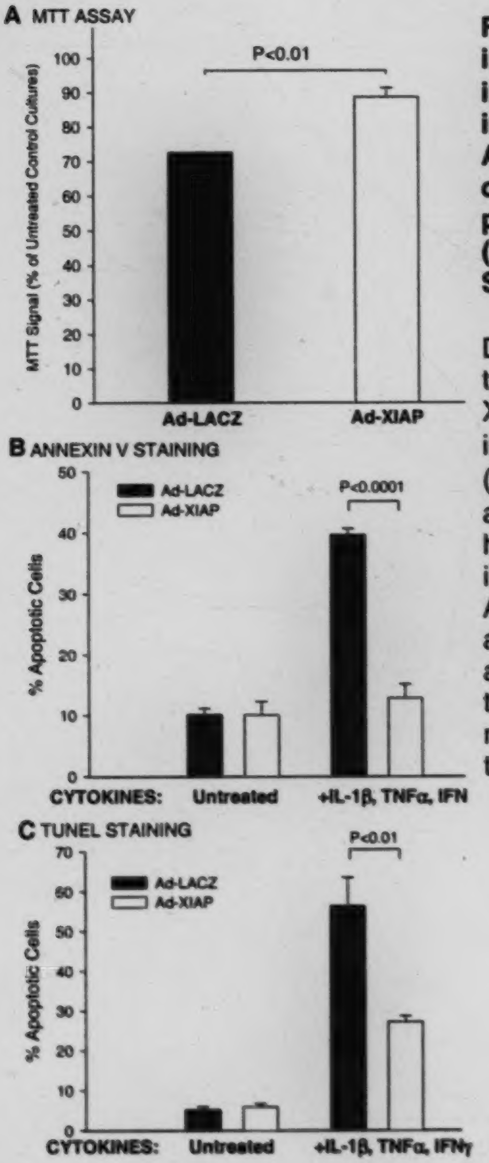

Figure 3-4: XIAP overexpression inhibits cytokine-induced apoptosis in βTC-Tet cells, as demonstrated by improved cell viability (MTT Assay, A; expressed as a % of non-treated control cultures) and decreased proportion of apoptotic cells (Annexin V Staining, B and TUNEL Staining, C).

Duplicate cultures of βTC-Tet cells were transduced with either Ad-LACZ or Ad-XIAP (10 V.P. per cell). After 48h incubation, a mixture of cytokines [IL-1β (Cf 10^2 U/mL), TNFα (Cf 10^3 U/mL), and IFNγ (Cf 10^3 U/mL)] were added to half of the culture dishes, and incubation continued for a further 72 h. Aliquots of cells from control (untreated) and cytokine treated culture dish were analyzed in triplicate using each of the three assays, and values are given as mean ±SE. Data are representative of three independent experiments.

3.3.4 Transduction of βTC-Tet cells with Ad-XIAP is associated with enhanced survival and more rapid reversal of diabetes following transplantation

To evaluate the role of apoptosis in the acute post-transplant period, XIAP- or LACZ-transduced βTC-Tet cells were transplanted in STZ-diabetic NOD-RAG$^{-/-}$ recipients (4×10^6 cells subcutaneously) as outlined in Fig. 3-5. We found that XIAP transduction significantly reduced the time required to achieve euglycemia (3.2±0.5 days, N=5) compared to control grafts expressing βGAL (normalized in 20.0±0.6 days, N=4) (Fig. 3-5B). The latter group was comparable to nontransduced βTC-Tet grafts, where time to normoglycemia was 21.3±3.4 days (N=5, Figure A1-1 in appendix 1). The superior survival characteristics of the XIAP transduced grafts required very early and continued administration of doxycycline to the recipients (20 mg/kg P.O. in food; this arrests the growth of the transplanted βTC-Tet cells and prevents mice from becoming hypoglycemic), as indicated by the arrow in Fig. 3-5A. Control βGAL grafts received doxycycline when euglycemia occurred at approximately three weeks. XIAP grafts (N=4) and βGAL grafts (N=2) remained functional for at least 50 days and performed similarly to control NOD-RAG$^{-/-}$ animals in glucose tolerance tests (Fig. 3-5C). (Note: Other animals in the cohort were lost to hypoglycemia or sacrificed for histology before 50 days, and hence they were not evaluated).

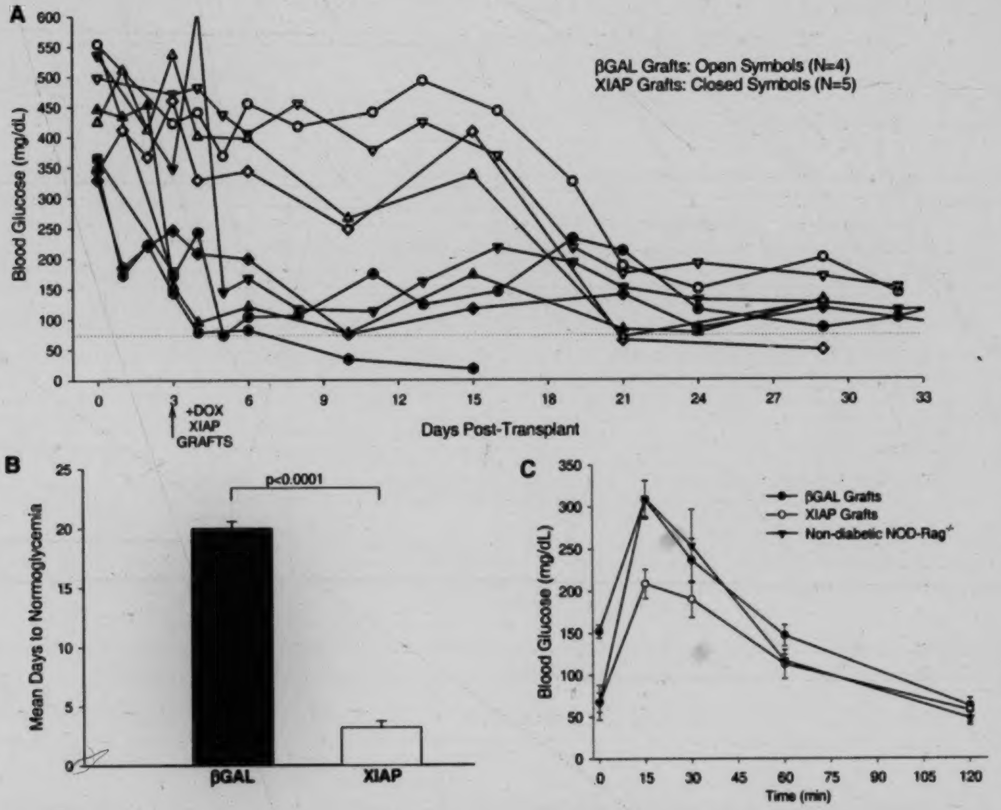

Figure 3-5: Transduction of βTC-Tet cells with Ad-XIAP is associated with enhanced survival and more rapid reversal of diabetes following transplantation subcutaneously into chemically-diabetic NOD-RAG$^{-/-}$ mice. Diabetes was induced in NOD-RAG$^{-/-}$ mice by streptozotocin injection. Six days later diabetic animals (blood glucose≥325 mg/dL) were transplanted with 4×10^6 Ad-LACZ transduced βTC-Tet cells (A, open symbols, N=4) or with the same number of Ad-XIAP transduced cells (A, closed symbols, N=5). The day of normoglycemia was defined as the first day where blood glucose was ≤220 mg/dL, provided that it remained ≤220 mg/dL on the next consecutive measurement. Animals that reached normoglycemia were maintained on doxycycline in order to arrest the growth of the transplanted βTC-Tet cells. Despite this, blood glucose levels did fall below 70 mg/dL (dotted line) in two animals, and these were euthanized (filled circle and open diamond; note also that 2 normoglycemic animals [open and closed upright triangles] were sacrificed for histology at 29 d). XIAP transduced cells achieved normoglycemia in 3.2±0.5 days, compared to 20.0±0.6 days for βGAL control grafts (B). Once animals had normalized and were on doxycycline diet, XIAP and LACZ transduced βTC-Tet grafts performed similarly to wild-type/endogenous NOD-RAG$^{-/-}$ islets, as measured by fasting glucose tolerance tests (C).

3.4 Discussion

In the present study, we found that inhibiting apoptosis via XIAP overexpression significantly enhanced β-cell survival during periods of stress due to hypoxia, reoxygenation, and cytokine insult. XIAP overexpression not only preserved β-cell viability, it allowed the β-cells to maintain appropriate responsiveness to glucose, the critical function that ordinarily disappears during stress. These results were confirmed by our finding that the mean time to achieve normoglycemia in a diabetic transplant recipient was decreased nearly 7-fold when XIAP was overexpressed within the β-cell graft (Fig. 3-5). These data represent the first instance where genetic modification of βTC-Tet cells resulted in improved engraftment in vivo, as measured by time to reverse hyperglycemia (Table 3-1).

Table 3-1: Summary of published results which utilize transplantation of unmodified or genetically modified βTC-Tet cells to reverse diabetes in mice, compared to results obtained in the current chapter

Cytoprotective gene *(stably transferred)*	# of cells transplanted	Transplant site	Recipient*	Median time to reverse hyperglycemia	Ref.
None	$2x10^6$	I.P.	STZ C3H	25 days	(20)
None	$1x10^7$	S.C./K.C.	STZ C57BL/6	25-35 days	(25)
Bcl-2	$2x10^6$	K.C.	STZ C3H	18-21 days	(11)
Bcl-2+MyD88	$2x10^6$	K.C.	STZ NOD-scid	25 days	(12)
Bcl-2+SOCS-1	$2x10^6$	K.C.	STZ NOD-scid	25 days	(12)
This Chapter					
None	$4x10^6$	S.C.	STZ NOD-$Rag^{-/-}$	21.3±3.4 days	
XIAP	$4x10^6$	S.C.	STZ NOD-$Rag^{-/-}$	3.2±0.5 days	
LACZ	$4x10^6$	S.C.	STZ NOD-$Rag^{-/-}$	20.0±0.6 days	

*Abbreviations: K.C.-kidney capsule, I.P.-intraperitoneal, S.C.-subcutaneous, STZ-streptozotocin treated. *Note that βTC-tet cells are syngeneic with C3H mice*

In the past, other molecules such as Bcl-2 (a mitochondria-associated anti-apoptotic protein), dominant negative MyD88 (blocks intracellular Il-1β signaling), and cFLIP (prevents caspase-8 activation) have been shown to inhibit hypoxia and/or cytokine mediated βTC-Tet cell death in vitro, with no apparent survival advantage conferred in vivo (11, 12, 26-28). We believe that the in vivo benefit of XIAP overexpression compared to these other anti-apoptotic molecules is directly related to its ability to potently block activation of late, effector apoptotic molecules (i.e. caspases 3 and 7), which prevents β-cell death in the multifaceted apoptotic milieu present in the immediate post-transplant period. Although levels of apoptosis in β-cells grafts was not measured directly, the rapid reversal of diabetes in animals receiving XIAP-transduced grafts suggests that apoptosis is a critical mediator of β-cell survival post-transplant.

Within the pancreas, islets are oxygenated by a glomerulus-like microvasculature that forms an intra-islet portal system, and during the process of islet isolation, this vasculature is completely disrupted, resulting in its degeneration and dedifferentiation (29, 30). As demonstrated in the present work, XIAP overexpression allows the βTC-Tet cells to survive in suboptimal conditions of oxygenation and nourishment. This finding suggests that XIAP could be used to enhance islet survival in culture prior to transplantation, which would improve clinical islet transplantation on several levels. First, suboptimal islet preparations could be cultured for longer periods, which would allow them to be pooled and transplanted at predictable times. Also, the frequent clinical situation where an islet preparation fails to meet transplant criteria due to low yields could be circumvented. Finally, the ability of XIAP to enhance islet survival during in vitro culture should make it far more practical to perform further gene therapeutic maneuvers, aimed at either conditionally immortalizing the β-cells (to create human β-cell lines), or alternately aimed at inducing the expression of immune-modulating molecules to mitigate allo- and autoimmune rejection.

One strategy to solve the problem of the limited supply of human islets involves the development of a renewal source of transplantable human β-cells. A number of groups are pursuing methods to generate β-cells from stem cells (embryonic or adult), pancreatic precursor cells, etc. with the goal of creating cell lines that would be clinically useful and bypass the need for cadaveric donor islets (reviewed in (31)). Regardless of the original cell source, differentiated and glucose responsive β-cells will by nature remain sensitive to hypoxia, hypoxia with reperfusion, and cytokine-mediated damage. Our results with XIAP provide a model of how a human β-cell line could be engineered to be resistant to these immediate post-transplant stresses.

Our current data demonstrates that in the context of improving the β-cell engraftment, XIAP expression may be required only transiently in the first few days or weeks following transplantation. This effect alone could greatly improve clinical islet transplantation by removing the current requirement for one or more 'booster' grafts. In rodent models, transplanted syngeneic islets become revascularized within 7-14 days but still only exhibit 15-20% of the oxygenation levels observed in endogenous pancreatic islets (4). The process of revascularization may be prolonged in diabetic patients who have experienced periods of uncontrolled hyperglycemia, further increasing the time to achieve optimal oxygenation of the transplanted tissue (32). We have demonstrated that the 50-70% decrease in β-cell mass induced by hypoxia and reoxygenation injury is completely prevented by XIAP overexpression (Fig. 3-2A). Any islets that manage to survive the physiological stress of engraftment are further challenged by a wave of proinflammatory cytokines produced by the recipient's innate immune system. In the present study XIAP overexpression prevented the 60-70% loss of β-cell mass observed in cytokine treated cultures (Fig. 3-4). It is important to note that in control cultures, whether treated under conditions of hypoxia, hypoxia with reperfusion, or cytokine challenge, any β-cells that survived had lost the ability to respond to glucose. In contrast, XIAP-expressing β-cells exposed to the same conditions recovered and retained most of their functional capacity (Fig. 3-3). XIAP-transduced cells required a recovery period of several days to restore the ability to respond to glucose following hypoxic insult, but they remained glucose responsive immediately following cytokine treatment (Fig. 3-3). This suggests that hypoxia and reoxygenation is a more potent apoptotic stimulus, most likely due to the multiple intracellular molecules that are upregulated during hypoxia and reoxygenation (i.e. ROS, NF-kB, etc.), which are known apoptotic effectors in β-cells. Based on these in vitro results, it appears that the protective effects of XIAP allow time for the islets to engraft in vivo and begin to produce insulin. We can also speculate that by reducing the total number of apoptotic β-cells in the graft, XIAP may reduce indirect immune stimulation due to host antigen presenting cells (APCs) presenting graft antigens.

Based on this premise and the ability of XIAP to block multiple apoptotic stimuli, we plan to assess the ability of XIAP to counteract cell-mediated destruction of βTC-Tet cells as well as its ability to block destruction of primary mouse and human islets in both allogeneic and/or autoimmune models. Should XIAP improve β-cell survival in these settings, it would hold promise for preventing β-cell death due to both physiological and immune-mediated stress. Our study demonstrates that XIAP overexpression greatly enhances β-cell survival during the early post-transplant period, by preventing apoptosis of βTC-Tet cells within subcutaneous grafts. Thus, XIAP is an important endogenous inhibitor of apoptosis, which--provided that methods can be developed to efficiently transduce most or all of the cells within whole intact islets--is likely to be very useful in promoting pancreatic β-cell survival following clinical islet transplantation for T1DM.

3.5 References

1. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000;343(4):230-8.

2. Pileggi A, Ricordi C, Alessiani M, Inverardi L. Factors influencing Islet of Langerhans graft function and monitoring. Clin Chim Acta 2001;310(1):3-16.

3. Moberg L, Johansson H, Lukinius A, Berne C, Foss A, Kallen R, et al. Production of tissue factor by pancreatic islet cells as a trigger of detrimental thrombotic reactions in clinical islet transplantation. Lancet 2002;360(9350):2039-45.

4. Carlsson PO, Palm F, Mattsson G. Low revascularization of experimentally transplanted human pancreatic islets. J Clin Endocrinol Metab 2002;87(12):5418-23.

5. Carlsson PO, Palm F, Andersson A, Liss P. Markedly decreased oxygen tension in transplanted rat pancreatic islets irrespective of the implantation site. Diabetes 2001;50(3):489-95.

6. Davalli AM, Scaglia L, Zangen DH, Hollister J, Bonner-Weir S, Weir GC. Vulnerability of islets in the immediate posttransplantation period. Dynamic changes in structure and function. Diabetes 1996;45(9):1161-7.

7. Moritz W, Meier F, Stroka DM, Giuliani M, Kugelmeier P, Nett PC, et al. Apoptosis in hypoxic human pancreatic islets correlates with HIF-1alpha expression. Faseb J 2002;16(7):745-7.

8. Rabinovitch A, Suarez-Pinzon WL. Cytokines and their roles in pancreatic islet beta-cell destruction and insulin-dependent diabetes mellitus. Biochem Pharmacol 1998;55(8):1139-49.

9. Biarnes M, Montolio M, Nacher V, Raurell M, Soler J, Montanya E. Beta-cell death and mass in syngeneically transplanted islets exposed to short- and long-term hyperglycemia. Diabetes 2002;51(1):66-72.

10. Ryan EA, Lakey JR, Paty BW, Imes S, Korbutt GS, Kneteman NM, et al. Successful islet transplantation: continued insulin reserve provides long-term glycemic control. Diabetes 2002;51(7):2148-57.

11. Dupraz P, Rinsch C, Pralong WF, Rolland E, Zufferey R, Trono D, et al. Lentivirus-mediated Bcl-2 expression in betaTC-tet cells improves resistance to

hypoxia and cytokine-induced apoptosis while preserving in vitro and in vivo control of insulin secretion. Gene Ther 1999;6(6):1160-9.

12. Cattan P, Rottembourg D, Cottet S, Tardivel I, Dupraz P, Thorens B, et al. Destruction of conditional insulinoma cell lines in NOD mice: a role for autoimmunity. Diabetologia 2003;46(4):504-10.

13. Klein D, Ribeiro MM, Mendoza V, Jayaraman S, Kenyon NS, Pileggi A, et al. Delivery of Bcl-XL or its BH4 domain by protein transduction inhibits apoptosis in human islets. Biochem Biophys Res Commun 2004;323(2):473-8.

14. Grey ST, Arvelo MB, Hasenkamp W, Bach FH, Ferran C. A20 inhibits cytokine-induced apoptosis and nuclear factor kappaB-dependent gene activation in islets. J Exp Med 1999;190(8):1135-46.

15. Grey ST, Longo C, Shukri T, Patel VI, Csizmadia E, Daniel S, et al. Genetic engineering of a suboptimal islet graft with A20 preserves beta cell mass and function. J Immunol 2003;170(12):6250-6.

16. Liston P, Roy N, Tamai K, Lefebvre C, Baird S, Cherton-Horvat G, et al. Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature 1996;379(6563):349-53.

17. Petrin D, Baker A, Coupland SG, Liston P, Narang M, Damji K, et al. Structural and functional protection of photoreceptors from MNU-induced retinal degeneration by the X-linked inhibitor of apoptosis. Invest Ophthalmol Vis Sci 2003;44(6):2757-63.

18. Trapp T, Korhonen L, Besselmann M, Martinez R, Mercer EA, Lindholm D. Transgenic mice overexpressing XIAP in neurons show better outcome after transient cerebral ischemia. Mol Cell Neurosci 2003;23(2):302-13.

19. Xu D, Bureau Y, McIntyre DC, Nicholson DW, Liston P, Zhu Y, et al. Attenuation of ischemia-induced cellular and behavioral deficits by X chromosome-linked inhibitor of apoptosis protein overexpression in the rat hippocampus. J Neurosci 1999;19(12):5026-33.

20. Efrat S, Fusco-DeMane D, Lemberg H, al Emran O, Wang X. Conditional transformation of a pancreatic beta-cell line derived from transgenic mice expressing a tetracycline-regulated oncogene. Proc Natl Acad Sci U S A 1995;92(8):3576-80.

21. Fleischer N, Chen C, Surana M, Leiser M, Rossetti L, Pralong W, et al. Functional analysis of a conditionally transformed pancreatic beta-cell line. Diabetes 1998;47(9):1419-25.

22. Sanes JR, Rubenstein JL, Nicolas JF. Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. Embo J 1986;5(12):3133-42.

23. Kilani RT, Mackova M, Davidge ST, Guilbert LJ. Effect of oxygen levels in villous trophoblast apoptosis. Placenta 2003;24(8-9):826-34.

24. Sladowski D, Steer SJ, Clothier RH, Balls M. An improved MTT assay. J Immunol Methods 1993;157(1-2):203-7.

25. Pericin M, Althage A, Freigang S, Hengartner H, Rolland E, Dupraz P, et al. Allogeneic beta-islet cells correct diabetes and resist immune rejection. Proc Natl Acad Sci U S A 2002;99(12):8203-6.

26. Cottet S, Dupraz P, Hamburger F, Dolci W, Jaquet M, Thorens B. SOCS-1 protein prevents Janus Kinase/STAT-dependent inhibition of beta cell insulin gene transcription and secretion in response to interferon-gamma. J Biol Chem 2001;276(28):25862-70.

27. Cottet S, Dupraz P, Hamburger F, Dolci W, Jaquet M, Thorens B. cFLIP protein prevents tumor necrosis factor-alpha-mediated induction of caspase-8-dependent apoptosis in insulin-secreting betaTc-Tet cells. Diabetes 2002;51(6):1805-14.

28. Dupraz P, Cottet S, Hamburger F, Dolci W, Felley-Bosco E, Thorens B. Dominant negative MyD88 proteins inhibit interleukin-1beta /interferon-gamma -mediated induction of nuclear factor kappa B-dependent nitrite production and apoptosis in beta cells. J Biol Chem 2000;275(48):37672-8.

29. Bonner-Weir S, Orci L. New perspectives on the microvasculature of the islets of Langerhans in the rat. Diabetes 1982;31(10):883-9.

30. Parr EL, Bowen KM, Lafferty KJ. Cellular changes in cultured mouse thyroid glands and islets of Langerhans. Transplantation 1980;30(2):135-41.

31. Itkin-Ansari P, Levine F. Sources of beta-Cells for Human Cell-Based Therapies for Diabetes. Cell Biochem Biophys 2004;40(3 Suppl):103-12.

32. Menger MD, Vajkoczy P, Leiderer R, Jager S, Messmer K. Influence of experimental hyperglycemia on microvascular blood perfusion of pancreatic islet isografts. J Clin Invest 1992;90(4):1361-9.

Chapter 4

XIAP Overexpression in Human Islets Prevents Early Post-Transplant Apoptosis and Reduces the Islet Mass Needed to Treat Diabetes

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4.1 Introduction

T1DM is a chronic, progressive autoimmune disorder that arises after the loss of the insulin-producing β-cells within pancreatic islets following the onset of autoimmunity. The recent introduction of the Edmonton Protocol has demonstrated that islet transplantation is a viable route to achieve insulin independence in a population of patients with T1DM (1). Despite its promise, islet transplantation remains restricted to patients with severe hypoglycemia or glycemic lability, and is presently unsuitable for the majority of patients with T1DM for several reasons. Most recipients require at least two islet transplant procedures (combined mass of >10,000 IE/kg body weight) in order to become insulin independent, which is a serious drawback given the prevalence of diabetes and the limited cadaveric organ donor pool (2, 3). The risks associated with islet transplantation appear to increase with the number of infusions and also with the total packed cell volume of cumulative grafts (4). Although the risks of post-transplant malignancy, lymphoid disorders, and sepsis have been low in islet-alone recipients, fears of these complications limit a broader application of clinical islet transplantation in patients with less severe forms of diabetes, especially children.

Expansion of clinical islet transplantation has been limited by the large requirement for donor tissue. Patients with chronic pancreatitis or pancreatic malignancies often have >50% of their pancreas removed without subsequent onset of diabetes, suggesting that only a fraction, perhaps 10-20%, of the estimated 10^6 endogenous islets may be necessary to maintain euglycemia in an individual (5). Thus, even if cadaveric islet isolations were only 20-50% efficient (which is often the case due to cold ischemia time, hemodynamic instability in the donor, variation in enzymatic activities, etc.), one might anticipate that insulin independence following single donor infusion would be routine, rather than the exception. The fact that most patients must receive >10,000 IE/kg to become insulin independent suggests that a large portion of the infused islets fail to engraft sufficiently. In fact, in murine models of islet transplantation it has been determined that even under ideal circumstances, >60% of syngeneic islet grafts are lost due to apoptosis (6). In clinical islet transplantation, it has been estimated that more than two thirds of the implanted islets never become functional (2).

This early, profound loss in islet mass can be attributed to several factors. Within a healthy pancreas, islet function is maximized by the intimate proximity of the β-cells and circulating blood, and as a result β-cells require a microenvironment with highly oxygenated blood (pO_2 of 40 mmHg) and abundant nutrients (7). The current method of human islet isolation and purification destroys the capillary network in islets, causing the rapid onset of hypoxia (8). Islet hypoxia immediately after transplantation into the portal circulation further extends the post-isolation hypoxic period (pO_2 of 5-10 mmHg or <1% O_2), and the revascularization process leads to reperfusion injury and death in islets (7). Thus, the majority of the islet graft rapidly fails to engraft after injection and undergoes apoptosis, which begins within hours post-transplant and continues for up to two weeks (9, 10). The immediate physiological burden faced by transplanted islets is also exacerbated by high levels of tissue factor expression in islets (11, 12). The Uppsala Group have demonstrated that this causes an IBMIR to transplanted islets, with platelet deposition and subsequent macrophage-mediated islet destruction, resulting in enhanced allo-antigen driven immunological attack and further islet apoptosis (11, 12).

Since the majority of the islet tissue is lost to apoptosis for the reasons listed above, intervention with anti-apoptotic agents may substantially enhance preservation of islet mass following transplantation (6). Both extracellular and intracellular pathways of apoptosis have been implicated in β-cell death, and many studies have described inhibition of a variety of apoptosis-associated proteins, including cFLIP (prevents caspase-8 activation), dominant-negative MyD88 (blocks Fas-associated death domain engagement), A20 (inhibits NF-κB activation), Bcl-2, and Bcl-XL (mitochondria-associated anti-apoptotic proteins) (13-19). Each of these strategies has proven to be effective in vitro in preventing apoptosis in islets following stimulation of a specific pathway. However, in terms of preventing islet apoptosis and graft loss in the early post-transplant period, results thus far have been largely disappointing. Only A20 has been shown to protect β-cells from cytokine- and Fas-mediated killing in vitro and to moderately reduce the islet mass required for syngeneic islet transplantation (17, 18). Despite extensive investigation of these apoptosis prevention strategies in rodent models of islet transplantation, none of these anti-apoptotic gene transfer strategies has been reported to be successful in enhancing human islet engraftment.

Taken collectively, these results suggest that the best strategy to enhance islet survival in the harsh post-transplant engraftment period would be to target the executioner caspases that function late in apoptosis, beyond the convergence point of both intracellular and extracellular signaling pathways. The IAP family of apoptosis inhibitors possesses a number of potent, endogenous anti-apoptotic genes, including XIAP, which binds to the active site of the main effector caspases 3, 7, and 9. Recent studies examining the effect of XIAP overexpression in islet transplantation have been very promising (20, 21). When XIAP was overexpressed in human islets, islet death and loss of function was prevented following treatment with the immunosuppressive drugs tacrolimus, sirolimus, and MMF in vitro (21). The protective effects of XIAP were further magnified in the Tet-regulatable murine β-cell line, βTC-Tet, following Ad-XIAP transduction (20). XIAP overexpression markedly enhanced β-cell survival and functional recovery during periods of stress due to hypoxia, reoxygenation, and cytokine insult in vitro (20). These results were confirmed in vivo by the finding that the mean time to achieve normoglycemia in recipient mice was decreased nearly 7-fold in XIAP overexpressing grafts, which represented the first report of genetically modified βTC-Tet cells exhibiting improved engraftment in vivo, as measured by time to reverse hyperglycemia (20). Based upon these positive results, the impact of adenoviral-mediated XIAP overexpression in human islets during periods of hypoxia, reperfusion, and during post-transplant engraftment was investigated in the present study.

4.2 Materials and Methods

4.2.1 Human Islet Isolation

Cadaveric donor pancreata were removed with prior informed written consent and stored in chilled University of Wisconsin solution or two-layer system before islet isolation. Islet isolation was performed as previously described for human islets (1, 22, 23). Immediately post-isolation, islets were quantified with dithizone and assessed for viability with ethidium bromide/Cyto Green staining using established methods (Invitrogen Canada)(24, 25). CMRL 1066 culture medium supplemented with calcium chloride (2.13 mM), Glutamine (2 mM), HEPES (20 μM), human serum albumin (0.625%), insulin (1.7 μM), nicotinamide (10 mM), selenium (8 nM), sodium pyruvate (5 mM), transferrin (5.5 mg/ L), trasylol (20000 IU), and zinc sulfate (16.7 μM) (all from Sigma-Aldrich, Oakville, ON) was used for all experiments, unless otherwise noted.

4.2.2 Amplification and purification of recombinant adenoviruses

The first generation recombinant adenoviral constructs used in these experiments have been previously described (26). Ad-LACZ or Ad-XIAP virus stocks were amplified using 293A cells. Each virus preparation was purified and concentrated using the Adeno-X purification system (BD Biosciences/Clontech, Mississauga, ON). These virus stocks were titred using standard plaque assays on 293[illegible] cells and stored as frozen aliquots at -70 °C.

4.2.3 Adenoviral Transduction of Human Islets

Human islets were obtained ≤1 h following the completion of the isolation procedure from the University of Alberta Clinical Islet Isolation Lab. Quantification of islet equivalents and assessment of purity were performed by the isolation lab personnel, using a standard diameter of 150 µm as previously described (24, 25). The islets were transduced at 10 V.P./cell (based upon the average cellular content in a human islet being roughly 10^3 cells/islet equivalent) with Ad-XIAP or Ad-LACZ for 1-2 h in a minimal volume of CMRL 1066 culture medium and incubated at 37 °C in 5% CO_2. After this period, additional culture medium was added to the islet-virus mixture and incubated an additional 24 h. Following transduction, the islets were washed twice with culture medium and incubated for at least 48 h prior to all in vitro assays or 7-10 days for transplant studies, with culture medium changes every 48 h.

4.2.4 Analysis of adenoviral transgene expression

Ad-XIAP expression- The presence of human XIAP protein (Hu-XIAP) was detected by immunoblot using anti-Hu-XIAP monoclonal antibody (Clone 48, BD Pharmingen, Mississauga, ON). Lysates were prepared from 500 islets 48 h following Ad-XIAP transduction in EDTA-free protease inhibitor cocktail (Roche) supplemented with 0.1% SDS. BCA assays were performed to determine the protein concentration in each sample (Pierce Chemical Co., Rockford, IL). 10 µg of total protein extract from each islet lysate was run on a 10% SDS-PAGE gel, and the separated proteins were subsequently transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Nonspecific protein interactions were prevented by blocking the membranes for one hour in PBS containing 0.05% Tween-20 (PBS-T) and 5% dry skim milk ("blocking buffer"). Next, the membranes were washed briefly in PBS-T and then incubated overnight with a 1:250 dilution of anti-XIAP in blocking buffer at 4 °C with mild agitation. After washing the membranes in PBS-T, they were incubated with peroxidase-labeled goat anti-mouse IgG for one hour (1:2500 dilution in blocking buffer; Amersham Pharmacia Biotech). To visualize Hu-XIAP protein bands, we used the ECL detection system (Amersham Pharmacia Biotech) with BioMax-MR film (Kodak).

Ad-LACZ expression- βGAL activity was assessed following 4 h of staining using the method outlined by Sanes et al. (27).

4.2.5 Adenovirus Quantification in Islet Supernatants

Aliquots of culture supernatant were harvested prior to adenovirus transduction, and at days 2, 4, 6, 8, and 10 post transduction. After each sample was collected, the islets were washed twice with PBS and resuspended in fresh culture medium. Samples of culture supernantants collected following Ad-LACZ transduction of human islets were transferred to murine A9 fibroblast cells and allowed to incubate for 10 h. After an additional 24 h the cells were stained for βGAL activity and quantified to determine the amount of infectious virus present in each supernatant sample.

4.2.6 In Vitro Hypoxia and Reoxygenation Studies

Human islets that had been previously transduced with Ad-XIAP or Ad-LACZ were cultured in <1% oxygen conditions to mimic the hypoxic environment in the early post-transplant period using a well established system known to induce widespread β-cell death (28, 29). 6-well plates containing 500 Ad-XIAP or Ad-LACZ transduced human islets/well were placed into a modular-incubator chamber (Billups-Rothenberg, DeMar, CA) at 37 °C. A controlled purge for 15 min. at 200 mL/sec resulted in the replacement of the surrounding air within the chamber with nitrogen containing 5% CO_2. It has been previously determined that the oxygen content within this system following the purge step is stable at a concentration of approximately 10 mmHg (≈1% O_2) (28). After a period of 24 h within the chamber, the plates were removed and either analyzed immediately or cultured for an additional 24 h under normal, non-hypoxic conditions (20% O_2, 5% CO_2, 37 °C) to simulate reperfusion injury. Islets cultured in either hypoxic or hypoxic/reoxygenation environments were compared to matched, transduced islets that had been cultured for the same time period under normal conditions.

4.2.7 Apoptosis assays

Apoptosis in control, Ad-LACZ, or Ad-XIAP transduced human islets following exposure to hypoxia or hypoxia with reoxygenation was quantified using TUNEL staining, which detects single-strand DNA breaks which are only present in the early phases of apoptosis. Control, hypoxic, or reoxygenated Ad-XIAP or Ad-LACZ transduced human islets were fixed in 4% paraformaldehyde for 30 min., embedded in agar, processed and embedded in paraffin (30). 10 µm sections were deparrafinized, rehydrated, and stained for human insulin to identify β-cells using guinea pig anti-insulin (1:1000, Dako, Carpinteria, CA) and labeled using Phycoerythrin-conjugated anti-guinea pig IgG (Jackson Immunoresearch, West Grove, PA). Immediately after this immunohistochemical staining, the apoptotic nuclei were labeled with FITC-dUTP (Roche) using the TdT enzyme (Deadend Fluorometric TUNEL System, Promega, Madison, WI). The stained slides were analyzed immediately using fluorescent microscopy. Each sample was stained in triplicate, and the number of TUNEL+ cells per islet cross section was determined. At least 50 islets from each slide were examined.

4.2.8 Glucose-Stimulated Insulin Release Assays

For each experimental condition, duplicate aliquots containing 500 islets were washed three times in low-glucose medium (DMEM containing 100 mg/dL D-Glucose supplemented with 10% FCS) followed by gravity sedimentation. The media was then aspirated and replaced with either low glucose medium or high glucose medium (DMEM containing 450 mg/dL D-Glucose supplemented with 10% FCS) and incubated for 2 h at 37 °C, 5% CO_2. Aliquots from the supernatant (250 μL) were analyzed in triplicate for human insulin content using a radioimmunoassay (Linco Research, St. Charles, MO). In each experimental condition, the fold stimulation was calculated by dividing the mean insulin released from islets cultured in high glucose medium by the mean insulin released from islets cultured in low glucose medium in parallel.

4.2.9 Animals

Immunodeficient NOD-RAG$^{-/-}$ mice (NOD.129S7(B6)-*Rag1*^{tm1Mom}/J) were obtained from Jackson Labs (Bar Harbor, ME) and housed under specific pathogen-free conditions. Ethical approval was obtained from the animal welfare committee at the University of Alberta, and all mice were cared for according to the guidelines of the Canadian Council on Animal Care.

4.2.10 Islet transplantation studies

Diabetes was induced in 8-10 week old male NOD-RAG$^{-/-}$ mice using a single intraperitoneal (I.P) injection of STZ (200 mg/kg, Sigma). Animals were considered to be diabetic after two consecutive blood glucose measurements ≥325 mg/dL. Grafts containing a mass of 1000 or 600 islets that had been quantified via dithizone staining the day prior to transplantation were independently re-counted immediately prior to transplantation, to assure consistency amongst suboptimal islet mass experiments. Islets were transplanted under the left kidney capsule in confirmed diabetic mice. In all transplantation studies, the nonfasting blood glucose levels were measured in each animal every day for the first week to establish time to engraftment and on alternating days thereafter using a One-Touch Ultra Glucometer (Johnson and Johnson, New Brunswick, NJ). Islet grafts were considered to be functional when the nonfasting blood glucose returned to normoglycemic levels (<220 mg/dL). Euglycemic animals from each cohort were selected randomly and the graft-bearing kidney was removed to establish that the human islet graft was functional, as determined by a return to hyperglycemia post-nephrectomy.

4.2.11 Human C-Peptide Assays

In order to determine the change in non-fasting serum human C-peptide levels following islet transplantation, serum was collected from post-transplant animals via tail vein bleeds each week. The serum human C-peptide levels for individual mice over time were quantified using an ultrasensitive human C-peptide ELISA kit (Alpco Diagnostics, Windham, NH), and each serum sample was assayed in triplicate.

4.2.12 Statistical Methods

SigmaPlot 2000 (SPSS, Inc. Chicago, IL, USA) was used for all statistical analyses in this study. Results are expressed as mean ± SEM. To compare results from in vitro hypoxia and hypoxia with reoxygenation experiments, student's *t*-tests were used.

4.3 Results

4.3.1 Expression of Ad-XIAP and Ad-LACZ in human islets

To study the effect of XIAP overexpression on β-cell survival, human islets were transduced with an adenoviral vector containing the human XIAP cDNA (Ad-XIAP), or a control vector containing the βGAL cDNA (Ad-LACZ). In both cases the transgene was driven by the chicken β-actin promoter, which gives stable, high levels of gene expression (26). Immunoblot analysis of Ad-XIAP transduced human islets revealed that following transduction, the islets expressed high levels of the recombinant human XIAP protein, which varied slightly amongst different preparations of islets (Fig.4-1A). Since the human XIAP gene was overexpressed in human islets, we were unable to quantify the number of Hu-XIAP-expressing islets by immunohistochemistry without generating a low background staining of endogenous XIAP protein. However, when a similarly grown and titred Ad-LACZ virus was used to transduce human islets in parallel at the same multiplicity of infection (m.o.i.) as Ad-XIAP, βGAL staining showed that

>95% of the islets present in the sample were transduced, at least on the surface (Fig. 4-1C), while untransduced human islets exhibited no evidence of βGAL staining (Fig. 4-1B). To determine the penetrance of the adenoviral transduction in human islets, X-GAL stained islets were embedded in agar and sectioned. As shown in Fig. 4-1E, most of the cell clusters were completely transduced by the Ad-LACZ vector. Immunohistochemical analysis for the ductal cell marker CK19 in X-GAL stained islets illustrated that the larger cell clusters with no visible staining in the middle were in fact ductal in origin, suggesting that all of the smaller, completely transduced clusters were islets (Fig. 4-1F). Immunohistochemical staining for insulin in these sections was not possible due to the destruction of insulin epitopes following the X-GAL staining procedure, but dithizone staining in Ad-LACZ or Ad-XIAP transduced islets consistently demonstrated >90% islet purity (see Fig. A1-2 in Appendix 1). Ad-XIAP or Ad-LACZ transduced human islets showed no loss in their ability to secrete insulin in response to glucose stimulation (Fig. 4-3, untreated controls).

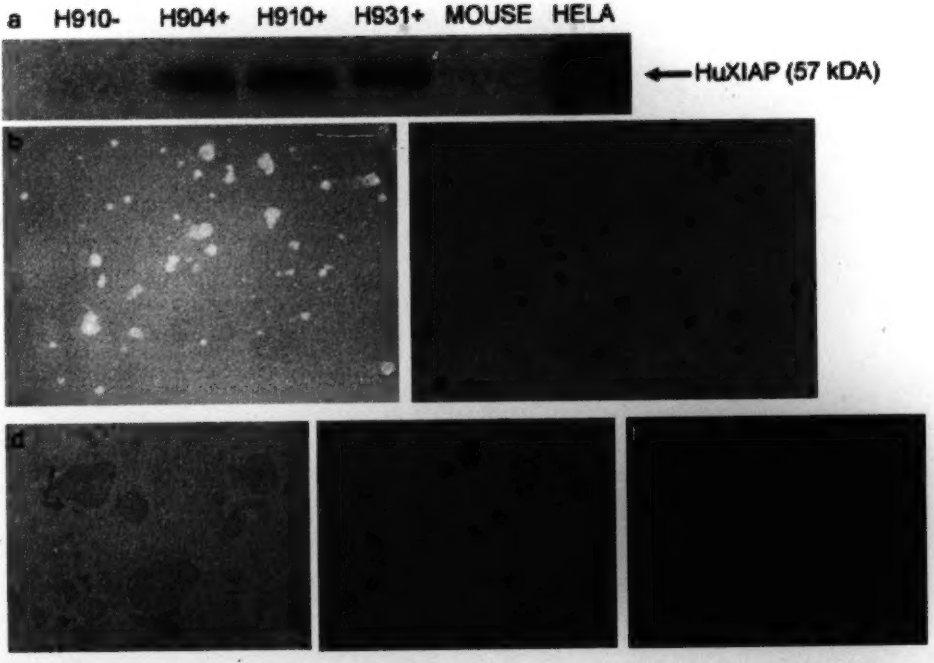

Figure 4-1: Adenoviral-mediated expression of Hu-XIAP or βGalactosidase in human islets.
(A) 10 µg of total protein from human islet lysates prepared from untransduced (H910) or Ad-XIAP transduced (H904+, H910+, H931+) human islets (three independent preparations) were run alongside lysates made from two control cell lines. As a negative control, untransduced murine βTC-Tet cells (MOUSE) were used, since they do not produce any detectable Hu-XIAP. As a positive control, the lane labeled 'HELA' contains 10 *µ*g protein lysate from Hela cells, which are known to express high levels of endogenous Hu-XIAP. Untransduced human islets from preparations H904 and H931 also show no detectable HuXIAP (see Fig. A1-3 in Appendix 1). Two days following Ad-LACZ transduction of human islets, X-GAL staining was performed. Nearly all of the Ad-LACZ transduced islets exhibited high levels of blue βGAL staining, as observed while in suspension (C; arrow designates one of the few islets that was poorly transduced), while untransduced islets exhibited no background staining (B). Penetrance of βGAL staining within intact islets was determined by sectioning agar-embedded islets following βGAL staining. The lower panels show untransduced (D) or Ad-LACZ transduced (E) human islets stained with X-GAL after 48 hours in culture. (F) Immunohistochemistical staining for human CK19, a ductal cell marker, in Ad-LacZ-transduced islets following X-GAL staining illustrates that the cell clusters which do not exhibit blue staining in the center in (E) are of ductal origin. Islets are shown at 40x magnification (B and C) or 100x magnification (D, E, and F).

4.3.2 Hypoxia and hypoxia-reoxygenation induced apoptosis is inhibited by XIAP overexpression

To examine the protective effects of XIAP during periods of hypoxia and following hypoxia-reoxygenation injury, a well-characterized, in vitro hypoxia-reperfusion model system that has proven to induce apoptosis in β-cells was utilized (28, 29). After being exposed to hypoxia for 24 h (<1% O_2, 5% CO_2), 53.75±4.84 cells/islet cross section in the LACZ transduced human islets stained TUNEL positive, while the levels of spontaneous apoptosis in the parallel, untreated control islets remained at 5.71±1.46 cells/islet cross section (Fig. 4-2). However, when XIAP was overexpressed in the islets during hypoxia, very few TUNEL positive apoptotic cells/islet section were observed (9.60±1.36 cells/islet cross section; Fig 4-2). When islets were subjected to 'reperfusion injury', the protective effect of XIAP was even more apparent, as the number of apoptotic cells in XIAP transduced islets (32.83±4.78 cells/islet cross section) was significantly reduced compared to Ad-LACZ transduced islets (171.825±9.71 cells/islet cross section) (Fig. 4-2B).

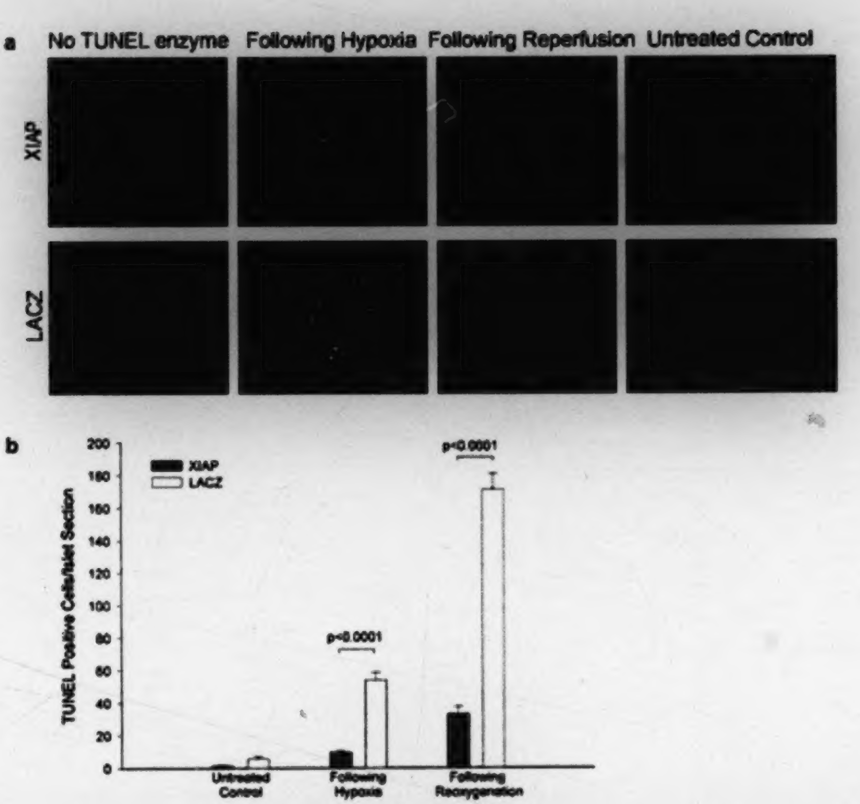

Figure 4-2: Hu-XIAP overexpression protects transduced human islets from hypoxia and hypoxia-'reperfusion' injury (i.e. re-oxygenation), as demonstrated by decreased numbers of apoptotic cells (TUNEL Staining).

Hypoxia was achieved in Ad-LACZ and Ad-XIAP transduced human islets by incubating plates in <1% O_2, 5% CO_2 for 24 h. Hypoxia with re-oxygenation ('reperfusion') was accomplished when islets maintained for 24 h in hypoxic conditions were transferred back to oxygenated conditions (20% O_2, 5% CO_2) for an additional 24 h. (A) Sections of embedded XIAP-transduced and LACZ-transduced islet were stained for insulin (labeled with phycoerythrin; shown in red) and TUNEL (labeled with FITC; shown in green). Representative islets from each experiment are shown at 400x magnification. As a control for nonspecific FITC-dUTP incorporation, sections were stained for insulin and treated with TUNEL label in the absence of TUNEL enzyme (A; "no TUNEL enzyme"). Islet sections treated with the phycoerythrin-conjugated secondary antibody in the absence of the anti-insulin primary antibody exhibited no detectable fluorescence (see Fig. A1-4 in Appendix 1). (B) The number of TUNEL positive cells per islet in each experimental condition was quantified (black bars represent Ad-XIAP transduced islets, and white bars represent Ad-LACZ-transduced islets). Slides were stained in duplicate, and at least 50 islets were examined from each section. The data are presented as mean±SEM and are representative of two independent experiments.

Although XIAP overexpression protected human islets from apoptotic death during hypoxia, it did not prevent the transient loss of β-cell function (Fig. 4-3). This result was confirmed by the low levels of insulin immunohistochemical staining in both control and XIAP-transduced islets, suggesting that hypoxia results in massive β-cell degranulation (Fig. 4-2A). Still, XIAP overexpression enhanced islet functional recovery following reperfusion, since XIAP transduced cells showed improved glucose-stimulated insulin secretion, while control LACZ-transduced islets were unable to respond to glucose stimulation after the 'reperfusion' (i.e. reoxygenation) period (Fig. 4-3).

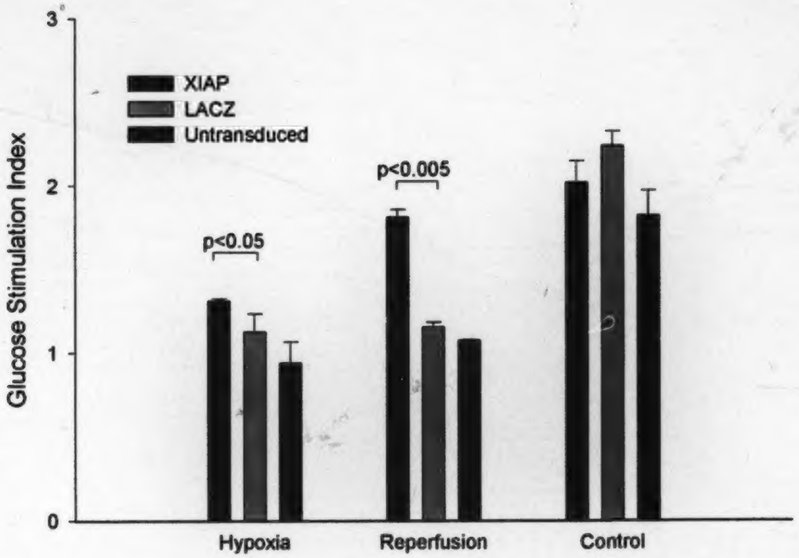

Figure 4-3: XIAP overexpression protects transduced human islets from hypoxia and hypoxia-'reperfusion' injury-induced loss of function, as demonstrated by glucose-stimulated insulin release assay.

Hypoxia was achieved in untransduced, Ad-LACZ and Ad-XIAP transduced human islets by incubating flasks in <1% O_2, 5% CO_2 for 24 h. When human islet cultures maintained for 24 h in hypoxic conditions were transferred back to oxygenated conditions ('reperfusion,' 20% O_2, 5% CO_2), glucose-stimulated insulin release assays were performed at time 0 ('following hypoxia') and 24 h ('following reperfusion'). Human insulin levels were measured in triplicate for each experimental condition, and the glucose stimulation index was calculated by dividing the mean insulin secretion in high glucose medium by the mean insulin secretion in low glucose medium (black bars represent Ad-XIAP transduced islets, and white bars represent Ad-LACZ-transduced islets). The data are presented as mean±SEM and are representative of three independent experiments using different islet preparations.

4.3.3 Presence of adenovirus in supernatants of cultured human islets following transduction

In the clinical context, transduction of islets ex vivo is attractive since the majority of the adenoviral vector could be washed away, in theory, prior to portal vein infusion, reducing the risk of non-targeted gene transfer to the recipient. This hypothesis has not been verified, so culture supernatants from Ad-LACZ transduced islets were collected and transferred to murine A9 fibroblast cells (which are very susceptible to AdV transduction), to determine if infectious virions were present for up to ten days post-transduction. Samples were collected on every second day after virus exposure, beginning on day 2 (at which point no washes had yet been performed after the addition of the vector on day 0). Following collection of the sample, the islets were washed twice with PBS and resuspended in fresh culture medium. Pre-transduction culture supernatant was used as a negative control, and had no infectious Ad-LACZ present, as indicated by the complete absence of βGAL staining in treated A9 cells. As expected, supernatants collected on day 2 had a high concentration of infectious Ad-LACZ, since none of the free virus had been washed away (Fig. 4-4). The amount of infectious Ad-LACZ in human islet culture supernatants decreased dramatically until no detectable βGAL staining was observed in A9 cells treated with day 10 supernatants (Fig. 4-4).

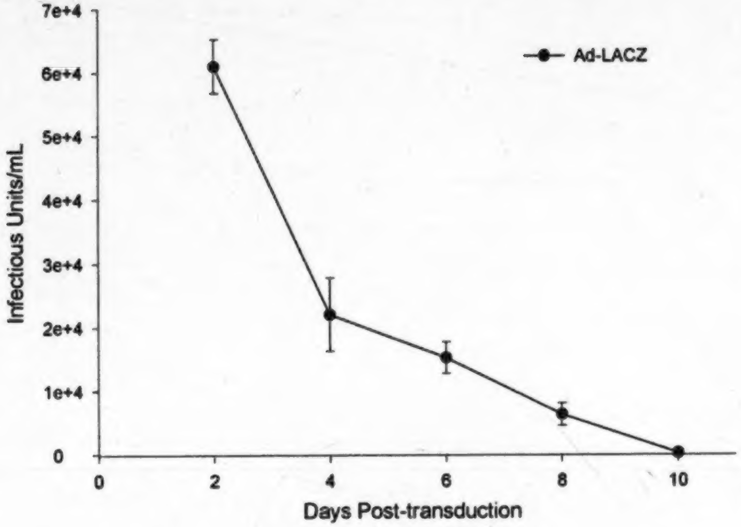

Figure 4-4: Adenovirus levels in supernatants of cultured human islets decrease significantly over 10 days in culture.
Culture supernatants from Ad-LACZ transduced islets were harvested pre-transduction, and at 2, 4, 6, 8, and 10 days post-transduction to measure the number of infectious particles present in supernatant samples, as determined by adding Ad-LACZ supernatant samples to murine A9 fibroblast cells and staining for βGAL expression after 48 h. Samples collected prior to the addition of adenovirus had no detectable infectious virus. Each sample was assayed in triplicate, and levels are expressed as mean±SEM.

4.3.4 Reversal of diabetes required transplantation of a significantly reduced number of Ad-XIAP transduced human islets

Based upon the observation that XIAP-transduced human islets were protected from hypoxia and hypoxia-reoxygenation death in vitro, we set out to evaluate the role of apoptosis in the acute post-transplant period in human islet transplantation. In our experience, a graft mass of 2000 IE/mouse is necessary to reverse hyperglycemia in diabetic mice, which is in keeping with other reports

(31-33). Marginal mass grafts containing 1000 or 600 XIAP- or LACZ-transduced human islets were transplanted in STZ-diabetic NOD-RAG$^{-/-}$ recipients (Fig. 4-5). Control grafts containing 1000 Ad-LACZ transduced human islets reversed hyperglycemia only 10% of the time (N=10, Fig. 5A), while grafts containing only 600 islets never became functional (N=7, Fig. 5A). In the presence of XIAP overexpression, islet grafts containing 600 islets were able to engraft rapidly and restore euglycemia in 89% of the diabetic mice (N=9, Fig. 5A). The superior survival characteristics of the XIAP transduced grafts were confirmed by measurement of serum human C-peptide levels in transplanted animals. Compared to serum from animals that had received 600 or even 1000 Ad-LACZ transduced human islets, the serum from animals receiving 600 Ad-XIAP transduced islets showed significantly higher levels of human C-peptide, and these levels remained constant for several weeks post-transplant (Fig. 5B; animals in the control LACZ cohorts were hyperglycemic and had to be euthanized prior to the 21 day time point).

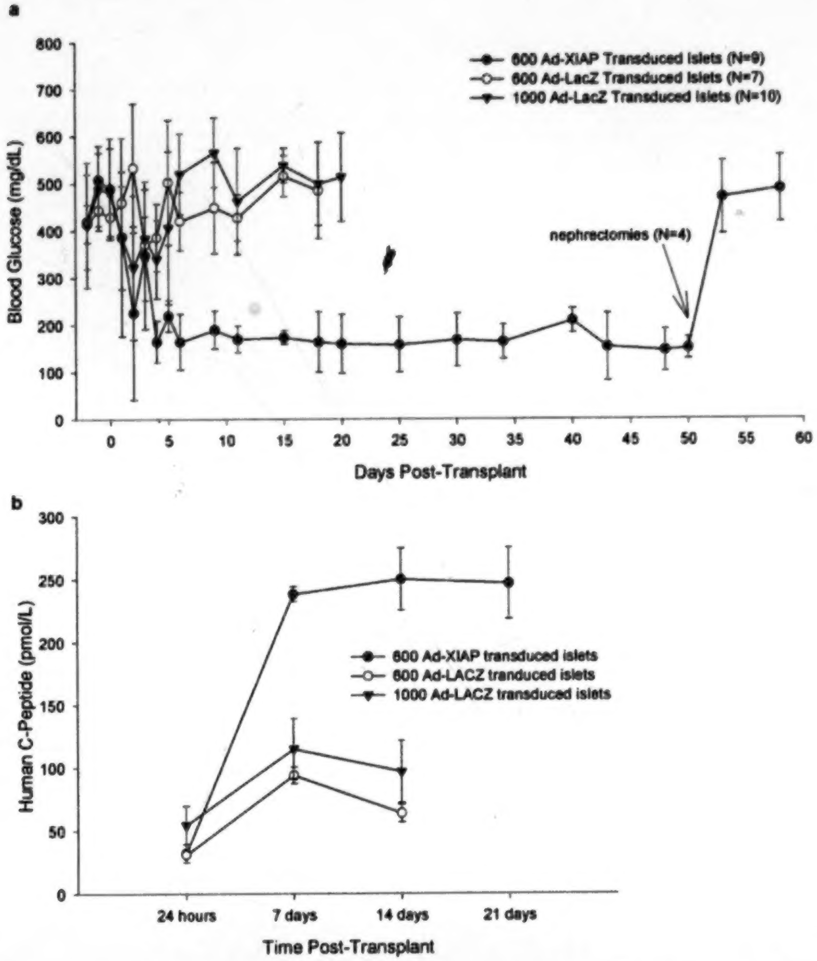

Figure 4-5: Significantly fewer XIAP-transduced human islets are required to reverse hyperglycemia when transplanted into diabetic NOD-RAG$^{-/-}$ mice.

(A) Diabetes was induced in NOD-RAG$^{-/-}$ mice by streptozotocin injection. 2-3 days later diabetic animals (blood glucose>18 mM/L) were transplanted with either marginal mass islet grafts containing 600 or 1000 Ad-LACZ transduced human islets (open circles (600 islets, N=7) and closed triangles (1000 islets, N=10)) or with 600 Ad-XIAP transduced cells (filled circles, N=9). 600 XIAP-transduced human islets provided enough β-cell mass to restore euglycemia in 89% of the animals, while none of the animals receiving 600 and 10% of the animals receiving 1000 LacZ control islets normalized (four independently islet donors were used in these experiments). (B) Animals receiving 600 XIAP transduced islets exhibited consistently high serum levels of human C-peptide compared to animals receiving βGAL control grafts with 600 or even 1000 islets.

4.4 Discussion

The present study demonstrates that XIAP overexpression enhances human islet survival during the post-transplant engraftment period by preventing hypoxia and hypoxia-reperfusion induced apoptosis. Inhibition of effector caspases via XIAP overexpression significantly enhanced human islet survival post-transplant, allowing a 70% reduction in human islet mass needed to reverse diabetes--the most profound reduction in graft mass capable of maintaining euglycemia in mice to be reported to date (Fig. 4-5). In an in vitro system that mimics hypoxia and reperfusion injury, XIAP-transduced human islets were significantly less apoptotic and recovered functional response to glucose stimulation following these insults, suggesting that XIAP mediates islet survival in vivo by inhibiting apoptosis during hypoxia and reperfusion stress (Figs. 4-2 and 4-3). These data confirm that the protective effect of XIAP overexpression previously observed in murine β-cells is reproducible and relevant in human islets.

The in vivo benefit of XIAP overexpression compared to other anti-apoptotic molecules is most likely related to its ability to potently block activation of late, effector apoptotic caspases, which prevents β-cell death in the multifactorial apoptotic environment present following portal vein infusion. This suggests that using XIAP in the clinical setting could immediately enhance the availability of islet transplantation by dramatically reducing the amount of islet tissue necessary to obtain insulin independence, effectively removing the need for multiple donor infusions. The recent announcement of a successful case of living-donor islet transplantation in Japan has provided renewed hope for many patients with type

1 diabetes (34). In order to bring living-donor islet transplantation to the mainstream and enhance recipient benefit for potential donor risk, success rates with marginal mass infusions must be improved, since only <50% of a donor's pancreas could be excised safely, and our data implies that potent apoptosis prevention with XIAP could have an immediate impact in these studies.

By reducing the total number of apoptotic islets within a graft, one can speculate that XIAP may reduce immune stimulation caused by host antigen presenting cells (APCs) presenting graft antigens. This is an important distinction from previously reported genetic manipulations to human islets that have moderately enhanced marginal mass graft survival, targeted at overexpression of β-cell growth factors like hepatocyte growth factor or erythropoietin, or overexpression of the insulin gene to enhance insulin output per islet (31, 35, 36). These strategies function by enhancing the survival, growth, and/or function of the islets that manage to engraft, but still exert little effect on apoptotic islets post-transplant. Thus, a large amount of donor antigen would theoretically still be presented to the recipient's immune system, potentially limiting the possibility of immunosuppressive therapy reduction or tolerance induction. Minimizing islet apoptosis post-transplant should prolong graft longevity, resulting in a more quiescent immunological state, and thereby enhance long-term rates of insulin-independence. If this could be achieved, islet transplantation would be potentially safer and therefore more available to a broader spectrum of patients with T1DM, including children.

The primary limitation in the clinical application of genetic modification to islets lies in the efficiency and reproducibility of gene transfer. Over the years, it has become apparent that adenoviral vectors represent the most reliable, efficient method to deliver genes to intact islets without the worrisome long term effect of non-targeted genomic integration associated with lentiviral vectors. That being said, many groups have struggled to obtain transduction efficiencies >50%, which limits the opportunity to observe any protective effect, especially with a transgene such as XIAP, which functions intracellularly (31, 36). One approach to improve transduction efficiency involves the use of more virus (i.e. increase the moi/cell), and while this does result in enhanced levels of transgene expression, the consequence is that the viral load itself becomes toxic to the islets (37). We hypothesized that transduction of islets very soon after isolation would enhance transduction efficiencies, since the capsule surrounding each islet would still be disrupted following enzymatic digestion. Through collaboration with the University of Alberta Clinical Islet Isolation Laboratory, we were able to obtain and transduce islets <1h following the completion of the isolation procedure. This resulted in highly efficient gene transfer to the islets, with >95% of Ad-LACZ stained islets exhibiting at least 75% X-GAL staining through the islet (Fig. 1E). This result is especially remarkable considering that only 10 V.P./cell was used, while other groups have obtained at most 50% transduction efficiency in human islets using 500-1000 V.P./cell (31, 36, 37). Even with the 50-100 fold decrease in adenovirus used to transduce the islets in our experiments, infectious virus was still detectable up to 8 days post-transduction, after more than twenty wash steps, suggesting that adenovirally transduced islets must be cultured for at least a week prior to transplantation to ensure that no vector is inadvertently transferred to the recipient (Fig. 4-4). Islet mass generally decreases in the culture setting due to hypoxic stress, which suggests that the use of an anti-apoptotic agent like XIAP would also enhance islet survival in this period.

Our studies suggest that effector caspase inhibition may be required only transiently to improve islet engraftment in the first few days or weeks following transplantation. Since first-generation adenoviral vectors were used in this study, and it is known that expression levels from these vectors declines 60-90 days post-transduction, we believe that XIAP overexpression would naturally taper off post-transplant, removing the need for the design of complex vectors with drug-regulatable promoters, for example (38). Our studies also suggest that XIAP gene transfer and overexpression could be circumvented altogether, should pharmaceutical agents targeted at preventing caspase activation become available, allowing the positive effects observed following XIAP overexpression to be reproduced using a transient drug therapy. Taken together, these data confirm that XIAP overexpression in human islets dramatically enhances engraftment and in so doing reduces the islet mass necessary to achieve euglycemia, suggesting that clinical application of this protocol could immediately and greatly enhance the availability and long-term outcome of islet transplantation for T1DM.

4.6 References

1. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000;343(4):230-8.

2. Ryan EA, Lakey JR, Paty BW, Imes S, Korbutt GS, Kneteman NM, et al. Successful islet transplantation: continued insulin reserve provides long-term glycemic control. Diabetes 2002;51(7):2148-57.

3. Shapiro J. Eighty years after insulin: parallels with modern islet transplantation. CMAJ 2002;167(12):1398.

4. Casey JJ, Lakey JR, Ryan EA, Paty BW, Owen R, O'Kelly K, et al. Portal venous pressure changes after sequential clinical islet transplantation. Transplantation 2002;74(7):913-5.

5. Slezak LA, Andersen DK. Pancreatic resection: effects on glucose metabolism. World J Surg 2001;25(4):452-60.

6. Biarnes M, Montolio M, Nacher V, Raurell M, Soler J, Montanya E. Beta-cell death and mass in syngeneically transplanted islets exposed to short- and long-term hyperglycemia. Diabetes 2002;51(1):66-72.

7. Carlsson PO, Palm F, Andersson A, Liss P. Markedly decreased oxygen tension in transplanted rat pancreatic islets irrespective of the implantation site. Diabetes 2001;50(3):489-95.

8. Pileggi A, Ricordi C, Alessiani M, Inverardi L. Factors influencing Islet of Langerhans graft function and monitoring. Clin Chim Acta 2001;310(1):3-16.

9. Davalli AM, Scaglia L, Zangen DH, Hollister J, Bonner-Weir S, Weir GC. Vulnerability of islets in the immediate posttransplantation period. Dynamic changes in structure and function. Diabetes 1996;45(9):1161-7.

10. Moritz W, Meier F, Stroka DM, Giuliani M, Kugelmeier P, Nett PC, et al. Apoptosis in hypoxic human pancreatic islets correlates with HIF-1alpha expression. Faseb J 2002;16(7):745-7.

11. Rabinovitch A, Suarez-Pinzon WL. Cytokines and their roles in pancreatic islet beta-cell destruction and insulin-dependent diabetes mellitus. Biochem Pharmacol 1998;55(8):1139-49.

12. Moberg L, Johansson H, Lukinius A, Berne C, Foss A, Kallen R, et al. Production of tissue factor by pancreatic islet cells as a trigger of detrimental thrombotic reactions in clinical islet transplantation. Lancet 2002;360(9350):2039-45.

13. Cottet S, Dupraz P, Hamburger F, Dolci W, Jaquet M, Thorens B. SOCS-1 protein prevents Janus Kinase/STAT-dependent inhibition of beta cell insulin gene transcription and secretion in response to interferon-gamma. J Biol Chem 2001;276(28):25862-70.

14. Cottet S, Dupraz P, Hamburger F, Dolci W, Jaquet M, Thorens B. cFLIP protein prevents tumor necrosis factor-alpha-mediated induction of caspase-8-dependent apoptosis in insulin-secreting betaTc-Tet cells. Diabetes 2002;51(6):1805-14.

15. Dupraz P, Rinsch C, Pralong WF, Rolland E, Zufferey R, Trono D, et al. Lentivirus-mediated Bcl-2 expression in betaTC-tet cells improves resistance to hypoxia and cytokine-induced apoptosis while preserving in vitro and in vivo control of insulin secretion. Gene Ther 1999;6(6):1160-9.

16. Dupraz P, Cottet S, Hamburger F, Dolci W, Felley-Bosco E, Thorens B. Dominant negative MyD88 proteins inhibit interleukin-1beta /interferon-gamma -mediated induction of nuclear factor kappa B-dependent nitrite production and apoptosis in beta cells. J Biol Chem 2000;275(48):37672-8.

17. Grey ST, Arvelo MB, Hasenkamp W, Bach FH, Ferran C. A20 inhibits cytokine-induced apoptosis and nuclear factor kappaB-dependent gene activation in islets. J Exp Med 1999;190(8):1135-46.

18. Grey ST, Longo C, Shukri T, Patel VI, Csizmadia E, Daniel S, et al. Genetic engineering of a suboptimal islet graft with A20 preserves beta cell mass and function. J Immunol 2003;170(12):6250-6.

19. Klein D, Ribeiro MM, Mendoza V, Jayaraman S, Kenyon NS, Pileggi A, et al. Delivery of Bcl-XL or its BH4 domain by protein transduction inhibits apoptosis in human islets. Biochem Biophys Res Commun 2004;323(2):473-8.

20. Emamaullee JA, Liston P, Korneluk RG, Shapiro AMJ, Elliott J. XIAP Overexpression in Islet Beta-cells Enhances Engraftment and Minimizes Hypoxia-Reperfusion Injury. Am J Transplant 2005;5(6):1297-305.

21. Hui H, Khoury N, Zhao X, Balkir L, D'Amico E, Bullotta A, et al. Adenovirus-Mediated XIAP Gene Transfer Reverses the Negative Effects of Immunosuppressive Drugs on Insulin Secretion and Cell Viability of Isolated Human Islets. Diabetes 2005;54(2):424-33.

22. Lakey JR, Warnock GL, Shapiro AM, Korbutt GS, Ao Z, Kneteman NM, et al. Intraductal collagenase delivery into the human pancreas using syringe loading or controlled perfusion. Cell Transplant 1999;8(3):285-92.

23. Tsujimura T, Kuroda Y, Avila JG, Kin T, Oberholzer J, Shapiro AM, et al. Influence of pancreas preservation on human islet isolation outcomes: impact of the two-layer method. Transplantation 2004;78(1):96-100.

24. Ricordi C, Gray DW, Hering BJ, Kaufman DB, Warnock GL, Kneteman NM, et al. Islet isolation assessment in man and large animals. Acta Diabetol Lat 1990;27(3):185-95.

25. Barnett MJ, McGhee-Wilson D, Shapiro AM, Lakey JR. Variation in human islet viability based on different membrane integrity stains. Cell Transplant 2004;13(5):481-8.

26. Liston P, Roy N, Tamai K, Lefebvre C, Baird S, Cherton-Horvat G, et al. Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature 1996;379(6563):349-53.

27. Sanes JR, Rubenstein JL, Nicolas JF. Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. Embo J 1986;5(12):3133-42.

28. Kilani RT, Mackova M, Davidge ST, Guilbert LJ. Effect of oxygen levels in villous trophoblast apoptosis. Placenta 2003;24(8-9):826-34.

29. Emamaullee JA LP, Korneluk RG, Shapiro AMJ, and Elliott JF. XIAP Overexpression in Islet Beta-cells Enhances Engraftment and Minimizes Hypoxia-Reperfusion Injury. Am J Transplant 2005;In Press.(Appended Manuscript #1.).

30. Street CN LJ, Rajotte RV, Shapiro AMJ, Kieffer TJ, Lyon JG, Kin T, Korbutt GS. Enriched Human Pancreatic Ductal Cultures Obtained from Selective Death of Acinar Cells Express Pancreatic and Duodenal Homeobox Gene-1 Age-Dependently. The Review of Diabetic Studies 2004;1:66-79.

31. Deng S, Vatamaniuk M, Lian MM, Doliba N, Wang J, Bell E, et al. Insulin gene transfer enhances the function of human islet grafts. Diabetologia 2003;46(3):386-93.

32. Bottino R, Balamurugan AN, Bertera S, Pietropaolo M, Trucco M, Piganelli JD. Preservation of human islet cell functional mass by anti-oxidative action of a novel SOD mimic compound. Diabetes 2002;51(8):2561-7.

33. Davalli AM, Ogawa Y, Ricordi C, Scharp DW, Bonner-Weir S, Weir GC. A selective decrease in the beta cell mass of human islets transplanted into diabetic nude mice. Transplantation 1995;59(6):817-20.

34. S. Matsumoto TO, Y. Iwanaga, H. Noguchi, H. Nagata, Y. Yonekawa, Y. Yamada, K. Fukuda, K. Tsukiyama, H. Suzuki, Y. Kawasaki, M. Shimodaira, K. Matsuoka, T. Shibata, Y. Kasai, T. Maekawa, A.M.J. Shapiro, K. Tanaka. Insulin Independence after living donor distal pancreatectomy and islet allo-transplantation. Lancet 2005;In Press.

35. Lopez-Talavera JC, Garcia-Ocana A, Sipula I, Takane KK, Cozar-Castellano I, Stewart AF. Hepatocyte growth factor gene therapy for pancreatic islets in diabetes: reducing the minimal islet transplant mass required in a glucocorticoid-free rat model of allogeneic portal vein islet transplantation. Endocrinology 2004;145(2):467-74.

36. Fenjves ES, Ochoa MS, Gay-Rabinstein C, Molano RD, Pileggi A, Mendez AJ, et al. Adenoviral gene transfer of erythropoietin confers cytoprotection to isolated pancreatic islets. Transplantation 2004;77(1):13-8.

37. Contreras JL, Wu H, Smyth CA, Eckstein CP, Young CJ, Seki T, et al. Double genetic modification of adenovirus fiber with RGD polylysine motifs significantly enhances gene transfer to isolated human pancreatic islets. Transplantation 2003;76(1):252-61.

38. Imperiale MJ, Kochanek S. Adenovirus vectors: biology, design, and production. Curr Top Microbiol Immunol 2004;273:335-57.

Chapter 5

Neonatal Porcine Islets Exhibit Natural Resistance to Hypoxia Induced Apoptosis

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5.1 Introduction

T1DM arises following the progressive destruction of the insulin-producing β-cells within pancreatic islets by autoreactive lymphocytes. The introduction of the Edmonton Protocol in 2000 confirmed that islet transplantation can provide a population of patients with T1DM excellent glycemic control and insulin independence (1). Expansion of clinical islet transplantation has been limited by the large requirement for donor tissue, since at least two islet transplant procedures (combined mass of >10,000 IE/kg body weight) are typically required to establish insulin independence in recipients, which presents a considerable obstacle given the prevalence of diabetes and the limited supply of suitable cadaveric donor organs (1-3).

In order to broaden the availability of islet transplantation to the majority of patients with T1DM, alternative sources of insulin-producing tissue must be identified. Pigs are particularly attractive as a xenogeneic islet donor since they are widely available, produce insulin that is functional in humans, and could be selected for certain donor characteristics. However, despite many studies aimed at optimizing adult pig islet isolation, no protocols have been established that generate consistent, reproducible islet yields (4-6). Also, following the isolation, adult pig islets are notoriously fragile in the tissue culture setting, limiting the possibility to pool multiple donor preparations or manipulate the islets in vitro prior to transplantation (4-7). Experimentation over the last decade using neonatal porcine islets (NPI) has suggested that the difficulties associated with adult pig islets could be avoided, since high yields of NPI can be easily and reproducibly isolated and maintained in culture for several weeks (8). Even though NPI are immature and contain less β-cell mass than adult islets, they are responsive to glucose challenge in vitro and can establish euglycemia in diabetic, immunodeficient animals following a 6-8 week in vivo maturation period (8). It has been demonstrated that microencapsulated NPI can be accepted long term in immune-competent xenogeneic murine recipients (9). Recent data have shown that NPI allografts transplanted intraportally into diabetic adult pigs reverse hyperglycemia in approximately one week (10). The xenoreactive immune response to non-encapsulated NPI in mice can be overcome with immunomodulation using a short course of anti-LFA-1 and anti-CD154 or anti-CD45RB or with anti-CD154 and donor specific transfusion (11, 12). Also, the generation of α1,3-galactosyltransferase-deficient pigs has provided a source of islet tissue lacking the major xenoantigens causing hyperacute rejection in pig-to-human xenotransplantation (13). Thus, NPI show considerable promise as a potential source of donor tissue in clinical islet transplantation.

The current necessity for large numbers of donor islets is directly related to the observation that most of the transplant islet mass fails to engraft properly, due to the sensitivity of β-cells to the stresses of isolation and the post-transplant engraftment period. It has been determined using murine models of islet transplantation that even under ideal circumstances, >60% of syngeneic islet grafts are lost due to apoptosis, a finding that has been corroborated by results obtained in clinical islet transplantation, where estimates have suggested that at least two thirds of the implanted islets never become functional (2, 14). The immediate impact of this early post-transplant graft loss is evident, since more islets must be implanted than are actually necessary to achieve insulin independence. Furthermore, it is likely that the large amount of apoptotic islet antigens priming the recipient's immune system adversely affect the ability to maintain islet mass or to taper immunosuppressive therapies.

Studies using mature rodent and human islets have demonstrated that the widespread apoptotic death occurring within transplanted islets during the early engraftment period can be attributed to several factors related to oxidative stress. The process of islet isolation and purification causes the rapid onset of hypoxia (15). After transplantation into the portal circulation, the post-isolation hypoxic period (pO_2 of 5-10 mmHg or <1% O_2) is extended for up to two weeks until revascularization occurs, which leads to reperfusion injury and further apoptosis in islets (16-19). The sensitivity of NPI to the stresses associated with the early post-transplant engraftment period is currently unknown. Since NPI represent a potential source of islets for clinical islet transplantation, and since post-transplant apoptosis is a key determinant of islet mass required to treat diabetes (and potentially of long-term anti-graft immunity), in the present study the sensitivity of NPI to hypoxia, hypoxia-reperfusion, and post-transplant apoptosis was investigated.

5.2 Materials and Methods

5.2.1 Cell Culture

βTc-Tet refers to a pancreatic β-cell line derived from an insulinoma of transgenic C3H mice; these mice express tetracycline-regulated SV40 large T antigen specifically within pancreatic β-cells (20, 21). βTc-Tet cells were maintained in DMEM supplemented with 10% fetal bovine serum, 450 mg/dL glucose, 1 mM Na-pyruvate, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen Canada, Burlington, Ontario) in 5% CO_2 at 37 °C. The cells were allowed to continuously proliferate in all studies.

5.2.2 Animals

Landrace-Yorkshire neonatal pigs were obtained from the University of Alberta Farm (Edmonton, AB). Immunodeficient NOD-RAG$^{-/-}$ mice (NOD.129S7(B6)-*Rag1*^{tm1Mom}/J) were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions. Ethical approval was obtained from the animal welfare committee at the University of Alberta, and all animals were cared for according to the guidelines of the Canadian Council on Animal Care.

5.2.3 Islet Isolation

NPI were isolated from 1-3 day old Landrace-Yorkshire neonatal pigs (1.5-2.0 kg) using the method developed by Korbutt et al. (8). The pancreas was removed from anesthetized animals following laparotomy and exsanguination and placed into Hanks' balanced salt solution (Invitrogen Canada). The pancreas was cut into small pieces, digested with 2.5 mg/mL collagenase (Sigma-Aldrich Canada, Mississauga, Ontario), and filtered through a 500 µm nylon mesh. The filtrate containing NPI was cultured for 7-10 days at 37 °C, 20% CO_2 (ordinary culture conditions) in Ham's F10 medium supplemented with 180 mg/dL glucose, 50 µM isolbutalmethylxanthine (IBMX, ICN Biomedicals, Montreal, PQ), 0.5% bovine serum albumin (BSA, fraction V, radioimmunoassay grade, Sigma Aldrich Canada), 2 mM L-glutamine, 3 mM $CaCl_2$, 10 mM nicotinamide, 100 U/mL penicillin, and 100 µg/mL streptomycin (the latter 5 reagents all from Invitrogen Canada, Burlington, Ontario). Adult porcine islets were obtained from 2 year old female Landrace-Yorkshire pigs (200 kg) purchased from the University of Alberta Farm, isolated using the same technique previously described for human islets, and cultured in the same medium as NPI (see above) (1, 22, 23). NOD-RAG$^{-/-}$ islets were isolated using established methods (24).

5.2.4 Islet Transplantation Studies

A single intraperitoneal (I.P.) injection of STZ (200 mg/kg, Sigma) was administered to 8-10 week old male NOD-RAG$^{-/-}$ mice to induce diabetes, and animals were considered to be diabetic after two consecutive blood glucose measurements ≥325 mg/dL. Grafts containing a mass of 500 syngeneic NOD-RAG$^{-/-}$ islets or 2000 NPI were transplanted under the left kidney capsule in confirmed diabetic mice. Animals were euthanized at 24 h, 48 h, and 7 days post-transplant, and the graft-bearing kidney harvested, placed in formalin, and processed for immunohistochemistical analysis.

5.2.5 In Vitro Hypoxia and Reperfusion Injury Model

NPI that had been maintained in ordinary culture conditions for 8-9 days were shifted to <1% oxygen conditions using a hypoxia chamber system developed by R.T. Kilani et al. (25). Briefly, 6-well plates containing 500 NPI/well were transferred to a modular-incubator chamber (Billups-Rothenberg, DeMar, CA) and placed inside a tissue culture incubator maintained at 37 °C. A controlled purge for 15 min. (200 mL/sec.) replaced the surrounding air within the chamber with nitrogen containing 5% CO_2. It has been determined previously that the final oxygen content within this system is 10 mmHg (≈1% O_2) (25). Following 24 h within the chamber, the plates were removed and either analyzed immediately or cultured for an additional 24 h under normal conditions (20% O_2, 5% CO_2, 37 °C) to simulate re-oxygenation injury. NPI exposed to hypoxia or hypoxia with reoxygenation were compared to control NPI cultured for the same time period under normal conditions.

5.2.6 Cell Viability Assays

Cell viability in metabolically active cells was measured by detecting the reduction of 3-(4,5-dimethylthiazolyl-2) 2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich Canada, Mississauga, Ontario) into a blue formazan product (26).

5.2.7 Apoptosis Assays

Apoptosis of NPI cells in vitro following exposure to hypoxia or hypoxia with reoxygenation or alternately in vivo within transplanted grafts was quantified using TUNEL staining, which detects single-strand DNA breaks present in the early phases of apoptosis (Dead-end Apoptosis Detection System, Promega, Madison, WI). Nuclear counterstaining with DAPI (4',6-diamino-2-phenylindol, Molecular Probes, Eugene, OR) was used to detect all cells present in the sample. For in vitro experiments, NPI were stained in suspension, applied directly to slides with coverslips, and analyzed immediately using fluorescent microscopy (UV excitation at 240-380 nm). Each sample was stained in triplicate, and at least 500 cells were counted per slide. Experimental samples were compared to non-treated control cultures to determine parallel rates of spontaneous apoptosis. A small aliquot of NPI was harvested from each in vitro experimental condition and fixed in 4% paraformaldehyde for 30 min., embedded in agar, processed and embedded in paraffin (27). For in vivo experiments, islet grafts were harvested, placed in formalin, processed and embedded in paraffin. Deparaffinized sections (10 μm) of embedded NPI or islet grafts were stained to identify β-cells using guinea pig anti-pig insulin antisera (1:1000, Dako, Carpinteria, CA) together with PE-conjugated goat anti-guinea pig IgG secondary antibody (1:200, Jackson Immunoresearch, West Grove, PA). Immediately following immunohistochemical staining for insulin, apoptotic nuclei were labeled with FITC-dUTP (Deadend Fluorometric TUNEL System, Promega, Madison, WI), and then slides counterstained with DAPI and analyzed by fluorescent microscopy.

5.2.8 Glucose-stimulated Insulin Release Assays

For each experimental condition, duplicate aliquots containing 500 NPI were incubated in low-glucose medium (RPMI supplemented with 2 mM L-glutamine, 0.5% BSA, and 50.4 mg/dL glucose; all from Sigma-Aldrich Canada) for 10 min.

The NPI were then washed twice with low glucose medium and resuspended in either low glucose medium or high glucose medium (RPMI supplemented with 2 mM L-glutamine, 0.5% BSA, and 360 mg/dL glucose) and incubated for 2 h at 37 °C, 5% CO_2. Aliquots containing 250 µL supernatant from each sample were analyzed in triplicate for porcine insulin content using a radioimmunoassay (Linco Research, St. Charles, MO). To calculate the fold stimulation for each experimental condition, the mean insulin released from cells cultured in high glucose medium was divided by the mean insulin released from cells cultured in parallel in low glucose medium.

5.2.9 Western Blot Analysis

Lysates were prepared from 500 NPI or adult porcine islets in EDTA-free protease inhibitor cocktail (Roche) supplemented with 0.1% SDS. The bicinchoninic acid (BCA) assay was used to determine protein concentration (Pierce Chemical Co., Rockford, IL) in each sample, and Western blot analysis performed by running 20 µg total protein extract on a 10% SDS-PAGE gel, followed by transfer to nitrocellulose (Bio-Rad Laboratories, Hercules, CA). Nonspecific antibody-protein interactions were blocked using PBS containing 0.05% Tween-20 (PBS-T) and 5% skim milk powder (blocking buffer). XIAP protein was detected using an anti-XIAP monoclonal antibody (Clone 48; BD Pharmingen, Mississauga, ON) at 1:250 dilution in blocking buffer. Lysates prepared from Hela cells, which naturally express high levels of XIAP, were used as a positive control. BCL-2 was detected using an anti-BCL-2 monoclonal antibody (Clone 124; Upstate, Lake Placid, NY) at 1:1000 dilution in blocking buffer. A positive control lysate prepared from Hela cells engineered to overexpress BCL-2 was generously provided by S. Wasilenko and M. Barry (University of Alberta, Edmonton, AB). Peroxidase-labeled goat anti-mouse IgG (1:2500 in blocking buffer, Amersham Pharmacia Biotech) was used to detect binding of each primary antibody to specific regions on the membrane. The ECL detection system (Amersham Pharmacia Biotech) was used with BioMax-MR film (Kodak) to visualize XIAP or BCL-2 protein bands. Blots were stained using Amido Black (Sigma-Aldrich Canada) to confirm that an equal amount of protein had been loaded in each lane.

5.2.10 Statistical Methods

SigmaPlot 2000 (SPSS, Inc. Chicago, IL, USA) was utilized for all statistical analysis in this study, and results are expressed as mean ± SEM. Student's *t*-tests for paired and unpaired data were used to compare results from in vitro hypoxia and hypoxia with reoxygenation experiments.

5.3 Results

5.3.1 NPI remain viable and do not undergo apoptosis following hypoxia

The susceptibility of NPI to apoptosis during periods of hypoxia and following hypoxia-reperfusion type insult was examined in vitro using a hypoxic chamber apparatus that has been shown to induce high levels of apoptosis in a murine β-cell line (βTC-Tet) and in primary human islets (28, 29). Following exposure to hypoxia ($<1\%$ O_2, 5% CO_2) for 24 h, >84% of NPI remained viable as measured by the MTT assay (Fig. 5-1A); this represents a modest and statistically insignificant decrease in signal as compared to untreated control NPI cultures. This minor loss in viability was reflected by a corresponding minimal increase in the number of apoptotic cells following hypoxia (10.57±1.60% TUNEL positive, versus untreated control cultures with 7.62±4.06% TUNEL positive; Fig. 5-1B). The apparent resistance of NPI to oxidative stress was further magnified when NPI were subjected to 24 h of hypoxia followed by 24 h of culture in normal oxygen conditions (20% O_2, 5% CO_2; this two-step incubation mimics hypoxia-reperfusion injury). Levels of cell viability and rates of apoptosis in NPI cultures were statistically insignificant compared to those of control, untreated parallel cultures (Fig. 5-1A and 5-1B). Experiments conducted using control βTC-Tet cells in parallel confirmed that the system was functional and could induce a loss of viability (Fig. 5-1A) and high levels of apoptosis (Fig. 5-1B) in β-cells following hypoxia and hypoxia with reperfusion. Results obtained from hypoxic, reperfused, and control NPI that had been embedded in agar, sectioned, and stained for apoptosis illustrate that there was no marked difference in the number of TUNEL positive cells from each experimental condition (Fig. 5-1C; representative islets from each condition are shown with insulin stained red, TUNEL positive cells stained green, and nuclei stained blue).

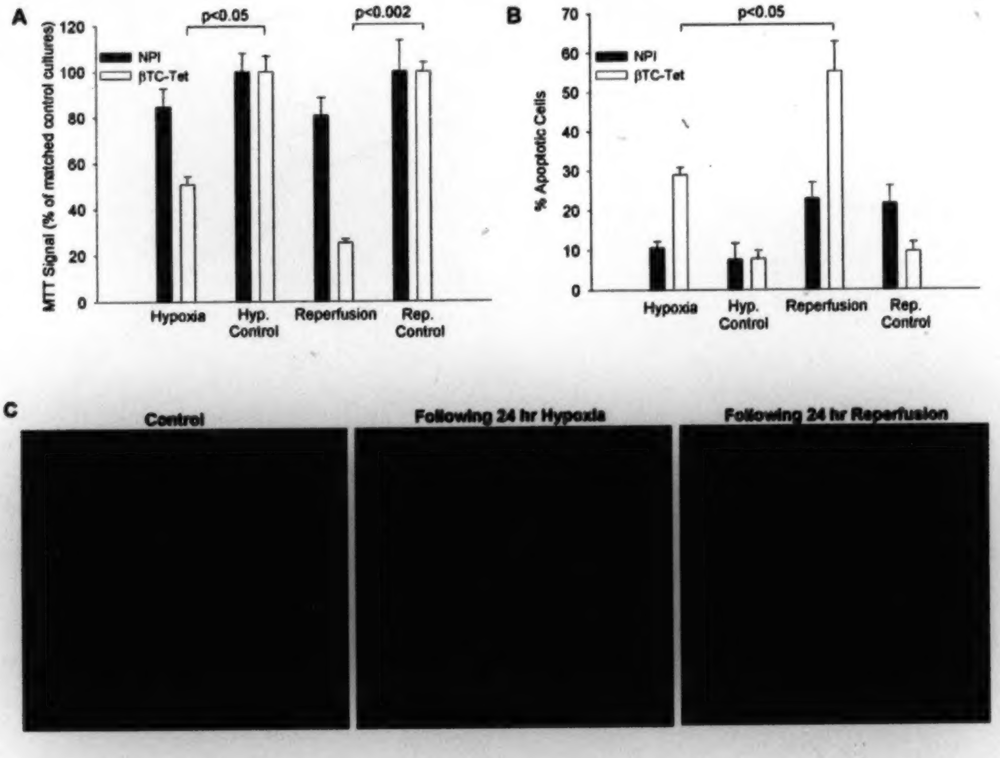


Figure 5-1: NPI are naturally resistant to loss of viability and apoptosis following hypoxia and hypoxia-'reperfusion' injury (i.e. re-oxygenation), as demonstrated by improved cell viability (MTT Assay, A) and decreased numbers of apoptotic cells (TUNEL Staining, B).

Hypoxia was achieved in NPI and βTC-Tet cells by incubating plates in <1% O_2, 5% CO_2 for 24h, while hypoxia with re-oxygenation was accomplished when cell cultures maintained for 24h in hypoxic conditions were transferred back to normal conditions (20% O_2, 5% CO_2) for a further 24h. Values are expressed as a % of matched control cultures, which were not subjected to hypoxia, but simply left in a standard tissue culture incubator. βTC-Tet cells were used as a control since it has been previously determined that this murine β-cell line is highly sensitive to hypoxia and reoxygenation in this system. The data are presented as mean±SE and are representative of two independent experiments, each performed in triplicate. (C) Sections of embedded NPI were stained for insulin (labeled with phycoerythrin; shown in red), TUNEL (labeled with FITC; shown in green), and all nuclei present (labeled with DAPI; shown in blue). Representative islets from each experimental condition are shown at 200x magnification.

5.3.2 NPI remain glucose responsive and continue to differentiate during hypoxia and following hypoxia-reoxygenation

The earliest indicator of β-cell stress, prior to the onset of apoptosis, involves the loss of glucose sensitivity. To examine the extent of β-cell function in NPI following hypoxia, hypoxia with reoxygenation, or in untreated control cultures, glucose-stimulated insulin release assays were performed immediately after each stimulus. As shown in Fig. 5-2A, there was no significant decrease in the glucose stimulation index immediately following hypoxia or reperfusion, compared to concurrent control NPI maintained in normal oxygen conditions. Thus the insulin secretory capacity of NPI exposed to hypoxia and hypoxia with reoxygenation increased at a rate that was comparable to parallel control cultures, which suggests that the penultimately differentiated cells within the NPI population retain the ability to differentiate into β-cells despite exposure to severe oxidative stress (Fig. 5-2B).

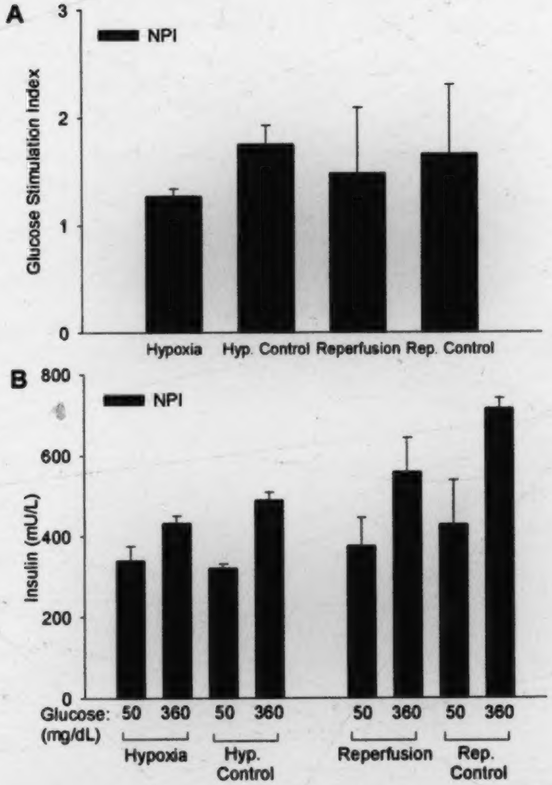


Figure 5-2: NPI remain glucose responsive and continue to differentiate following periods of hypoxia and reoxygenation.

Hypoxia was established incubating NPI in <1% O_2, 5% CO_2 for 24h. When NPI maintained for 24h in hypoxic conditions were transferred back to normal tissue culture conditions ("reperfusion," 20% O_2, 5% CO_2), glucose-stimulated insulin release assays were performed at 0h and 24h. Concurrent NPI maintained in normal tissue culture conditions were analyzed at the same time points as hypoxic cultures ('Hyp. Control') and reoxygenated cultures ('Rep. Control'). Insulin levels were measured in triplicate for each experimental condition, and the glucose stimulation index was calculated by dividing the mean insulin secretion in high glucose medium by the mean insulin secretion in low glucose medium (A). The insulin concentrations provided in (B) demonstrate that NPI continue to differentiate at the same rate as parallel control cultures, despite exposure to hypoxia and reperfusion. The data are presented as mean±SEM and are representative of three independent experiments using different NPI preparations.

5.3.3 NPI are less apoptotic during the post-transplant engraftment period, compared to syngeneic adult mouse islets

To investigate the resistance of NPI to hypoxia-induced apoptosis in vivo, NPI were transplanted into chemically diabetic, immuodeficient NOD-RAG$^{-/-}$ mice and compared to syngeneic NOD-RAG$^{-/-}$ islet grafts, since it has been previously determined that more than 60% of the islet mass is lost to early engraftment apoptosis even in syngeneic models (14). NPI or syngeneic islet grafts were harvested at 24 h and 7 days post-transplant in each cohort (N=3 at each time point) and processed for immunohistochemical analysis (Fig. 5-3; insulin staining in red, TUNEL staining in green, and nuclei staining in blue). As expected, syngeneic mouse islet grafts were largely apoptotic 24 h post-transplant, with many cells within the graft co-staining positive for insulin and TUNEL (Fig. 5-3C, yellow staining). After one week, when the remaining islets had engrafted, there was very little evidence of TUNEL positive apoptotic cells (one indicated with an arrow in Fig. 5-3D). NPI grafts exhibited low levels of insulin staining at both 24 h and 7 days post-transplant, which is consistent with the finding that NPI generally require 6-8 weeks to fully differentiate into a β-cell dense tissue (red insulin staining, Fig. 3A and 3B) (8). However, there was little evidence of either single TUNEL positive cells (shown in green) or insulin and TUNEL positive cells (shown in yellow; one indicated with an arrow in Fig. 5-3A) within NPI grafts 24 h or 7 days post-transplant (Fig. 5-3B).

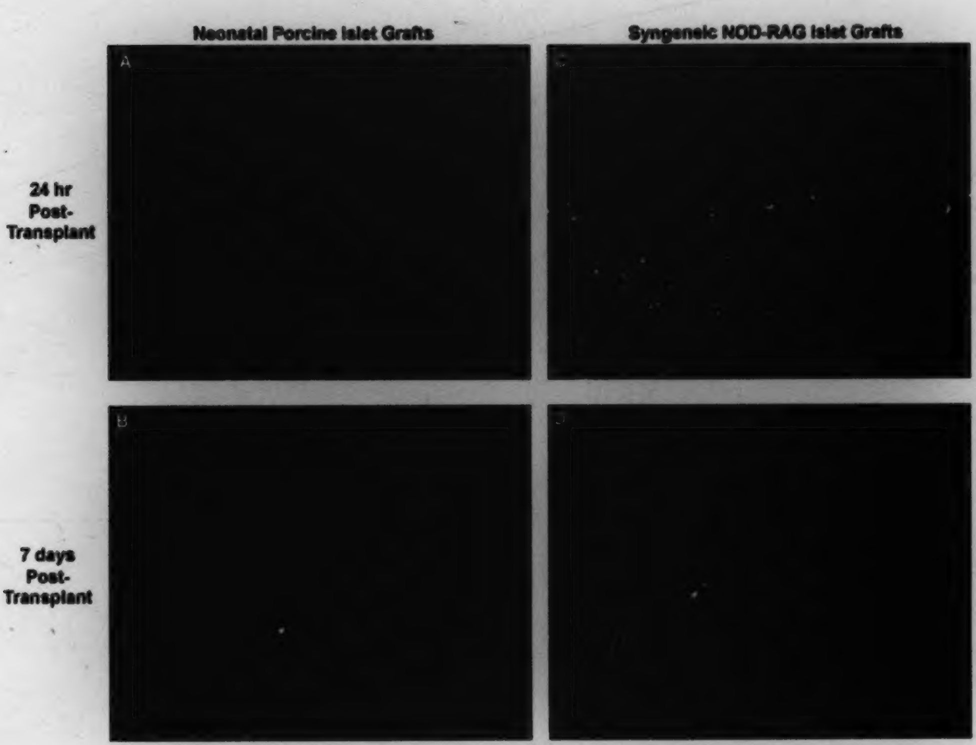


Figure 5-3: NPI are resistant to apoptosis during the post-transplant engraftment period.

NPI or syngeneic islets were transplanted under the kidney capsule of diabetic NOD-RAG$^{-/-}$ mice (STZ-induced). At 24 h and 7 days post-transplant, graft-bearing kidneys were harvested and analyzed for insulin staining (labeled with phycoerythrin; shown in red), TUNEL (labeled with FITC; shown in green), and all nuclei present (labeled with DAPI; shown in blue). For cells that are both insulin and TUNEL positive, the resulting color is yellow (co-staining with red and green). As shown in (A), very few TUNEL positive cells are present 24 h post-transplant in NPI grafts (one yellow cell identified with white arrow), whereas a large number of TUNEL positive β-cells are present in syngeneic murine islet grafts (expansive yellow staining in C). Grafts analyzed 7 days post-transplant demonstrate that both NPI (B) and syngeneic murine islets (D) are largely insulin positive (stained in red), with very little evidence of TUNEL positive apoptotic cells (white arrow identifies a yellow insulin/TUNEL positive cell in (D)). Three transplanted animals in each cohort were sacrificed at the indicated time points, and the immunohistochemical data shown is a representative example (100x magnification).

5.3.4 NPI naturally express high levels of the potent anti-apoptotic protein XIAP

To understand the mechanism by which NPI are resistant to apoptosis compared to adult porcine islets (the latter are highly fragile and survive poorly in culture), lysates were prepared from NPI and adult porcine islets and analyzed by Western blot to determine expression levels of two different anti-apoptotic proteins: BCL-2 and XIAP. BCL-2 is a potent inhibitor of mitochondrial-mediated apoptosis, which is the main intracellular apoptosis pathway implicated during oxidative stress-induced injury, while XIAP is an endogenous inhibitor of effector caspases that function downstream of both extracullar (receptor-mediated) and intracellular apoptotic pathways. Three independent NPI and adult porcine islet donor preparations were examined, and neither NPI nor adult porcine islets expressed any detectable BCL-2 protein (Fig. 5-4). In contrast, NPI consistently exhibited high levels of XIAP expression, compared to a complete absence of detectable XIAP protein in adult porcine islets (representative blot shown in Fig. 5-4).

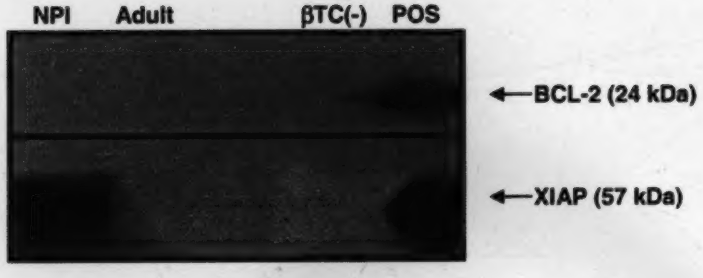


Figure 5-4: NPI naturally express high levels the potent anti-apoptotic gene XIAP, while adult porcine islets do not.

Cell lysates (20 μg total protein) prepared from NPI ("NPI") or Adult Pig Islets ("Adult Pig") were run alongside control lysates prepared from βTC-Tet cells ("βTC"; negative control) and Hela cells ("POS"; positive control). The Western blot was probed with an anti-XIAP monoclonal antibody, and the XIAP band of the expected size (57 kDa) appears in NPI as well as Hela cells, which naturally express high levels of XIAP. When Western blots prepared with the same NPI and adult porcine islet lysates were probed with an anti-BCL-2 monoclonal antibody, no BCL-2 protein was detected in either sample. A lysate prepared from Hela cells transfected with BCL-2 was used as a positive control for BCL-2 expression. Similar results were obtained in three independent experiments using three different preparations of NPI and adult pig islets.

5.4 Discussion

The profound loss of mature islet mass in the early post-transplant engraftment period can be attributed to the onset of apoptosis due to the exquisite sensitivity of β-cells to hypoxia and reperfusion injury. This study represents the first examination of the effects of oxidative stress on NPI, a tissue that has the potential to be used as an alternative, widely available β-cell source in clinical islet transplantation for T1DM. NPI were exposed to hypoxia and reoxygenation using an in vitro chamber model system which we have previously shown can induce significant loss of β-cell viability, loss of glucose responsiveness, and loss of overall mass due to apoptosis in both murine β-cells and human islets (28, 29). Surprisingly, NPI remained viable and did not become apoptotic during periods of hypoxia or even following reoxygenation (Fig. 5-1). In fact, NPI continued to secrete insulin in response to glucose, the critical function that normally disappears during periods of stress, regardless of exposure to hypoxia or reoxygenation (Fig. 5-2). These data challenge the long held belief that sensitivity to oxidative stress is an inherent characteristic in all islets, regardless of species of origin or degree of maturity.

Several results in our study provide insight into the maturation process of NPI in the context of islet transplantation. First, data shown in Fig. 5-2B illustrates that the β-cell mass and/or insulin secretory capacity in NPI continues to increase over time, irrespective of exposure to hypoxia or reoxygenation, suggesting that the stress of post-transplant engraftment does not have any deleterious effect on NPI maturation. Also, there was little evidence of apoptosis in NPI grafts 24 h post-transplant (in either the insulin positive β-cells or the other cells present around the β-cells in the graft), compared to mature islets, which supports data obtained in our in vitro analysis (Figs. 5-1 and 5-3). By one week post-transplant, there are virtually no detectable apoptotic cells in NPI grafts, a result that has been observed by other groups (30). Since less NPI tissue is dying after transplantation compared to intact islets, there is less apoptotic donor antigen present to be cleared by recipient antigen presenting cells and presumably less priming of the recipient's xenoreactive immune response. This is particularly important considering that transplanted islets are a fraction of the volume of donor tissue being implanted into a recipient compared to solid organ transplantation, and in this regard hold greater potential for tolerance induction, especially when the transplanted tissue remains highly viable post-transplant. The inherent resistance of NPI to post-transplant hypoxia and reperfusion injury makes them particularly attractive from this standpoint.

When we investigated the mechanism by which NPI exhibit robust survival in the face of significant stress, we found that NPI naturally express high levels the potent anti-apoptotic gene XIAP, while islets from adult pigs do not (Fig. 5-4). This observation is consistent with the findings of Liggins et al., who observed that fetal human islets overexpress another IAP family member, survivin, while adult human islets do not (31). This idea may relate to results obtained in Fig. 5-3, where within NPI grafts 24 h post-transplant, apoptosis is only observed to occur in cells which stain most strongly for insulin. It may be that in these highly differentiated β-cells, XIAP (and other IAPs) are no longer present at high enough levels to prevent programmed cell death (i.e. in a manner similar to adult pig islets, which are know to be sensitive to many pro-apoptotic stimuli). A corollary to this idea is that the reason NPI are able to resist apoptosis relates to the fact that they contain a large population of pen-ultimately differentiated 'pre-β-cells'. These cells still express high levels of IAPs (including XIAP), but they are nevertheless poised to rapidly differentiate into fully functional β-cells, and they continue to do so even after a hypoxic insult (consistent with the results shown in Fig. 5-2).

IAP overexpression in non-cancerous cells has only been observed in a few tissues, and appears to be temporally linked to specific developmental changes in these tissues (31-33). A temporal fall in the expression levels of potent endogenous anti-apoptotic proteins in maturing islets may explain the observation that a neonatal wave of β-cell apoptosis occurs in the first weeks of life in rodents (reviewed in (34)). The enhanced survival of NPI during periods of oxidative stress cannot be fully explained by XIAP overexpression alone, since we have previously reported that although XIAP overexpression in murine β-cells and human islets potently inhibits hypoxia and hypoxia/reoxygenation-induced apoptosis, the functional response to glucose is transiently lost and recovers only slowly during the reoxygenation period (28, 29). The fact that NPI are non-apoptotic and remain glucose responsive suggests that the mechanism of NPI survival is multifaceted and complex.

In conclusion, our data indicate that NPI possess a natural resistance to hypoxia and reoyxgenation-induced apoptosis and loss of function in vitro, findings that were confirmed by our observation that NPI grafts had very little evidence of apoptosis during the post-transplant engraftment period (24 h) or thereafter (7 days). The robust survival of NPI compared to adult islets could be attributed in part to the overexpression of XIAP, a powerful inhibitor of effector caspases that functions late in apoptosis. However, there are probably additional factors that contribute to the heartiness of NPI, and further investigation into the mechanisms acting may well suggest new therapeutic strategies to enhance the survival of adult islets in the transplant setting. The resistance of NPI to apoptosis post-transplant suggests that less donor antigen is released from NPI grafts compared to adult islet grafts, and as such NPI grafts may prove to be less immunogenic to the recipient. This concept is supported by the recent data reported by Kin et al., where ~30% of NPI allografts were spontaneously accepted in adult pigs in the absence of immunosuppression (10). Thus, NPI represent an attractive alternative source of tissue to expand the availability of clinical islet transplantation, due to their nearly limitless availability, ease of preparation, and enhanced survival characteristics both in vitro and in vivo, as demonstrated by the present study.

5.5 References

1. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000;343(4):230-8.

2. Ryan EA, Lakey JR, Paty BW, Imes S, Korbutt GS, Kneteman NM, et al. Successful islet transplantation: continued insulin reserve provides long-term glycemic control. Diabetes 2002;51(7):2148-57.

3. Shapiro J. Eighty years after insulin: parallels with modern islet transplantation. CMAJ 2002;167(12):1398.

4. Ricordi C, Socci C, Davalli AM, Staudacher C, Baro P, Vertova A, et al. Isolation of the elusive pig islet. Surgery 1990;107(6):688-94.

5. Ricordi C, Socci C, Davalli AM, Staudacher C, Vertova A, Baro P, et al. Swine islet isolation and transplantation. Horm Metab Res Suppl 1990;25:26-30.

6. Toso C, Brandhorst D, Oberholzer J, Triponez F, Buhler L, Morel P. Isolation of adult porcine islets of Langerhans. Cell Transplant 2000;9(3):297-305.

7. Socci C, Ricordi C, Davalli AM, Staudacher C, Baro P, Vertova A, et al. Selection of donors significantly improves pig islet isolation yield. Horm Metab Res Suppl 1990;25:32-4.

8. Korbutt GS, Elliott JF, Ao Z, Smith DK, Warnock GL, Rajotte RV. Large scale isolation, growth, and function of porcine neonatal islet cells. J Clin Invest 1996;97(9):2119-29.

9. Omer A, Duvivier-Kali VF, Trivedi N, Wilmot K, Bonner-Weir S, Weir GC. Survival and maturation of microencapsulated porcine neonatal pancreatic cell clusters transplanted into immunocompetent diabetic mice. Diabetes 2003;52(1):69-75.

10. Kin T, Korbutt GS, Kobayashi T, Dufour JM, Rajotte RV. Reversal of diabetes in pancreatectomized pigs after transplantation of neonatal porcine islets. Diabetes 2005;54(4):1032-9.

11. Rayat GR, Gill RG. Indefinite survival of neonatal porcine islet xenografts by simultaneous targeting of LFA-1 and CD154 or CD45RB. Diabetes 2005;54(2):443-51.

12. Appel MC, Banuelos SJ, Greiner DL, Shultz LD, Mordes JP, Rossini AA. Prolonged survival of neonatal porcine islet xenografts in mice treated with a donor-specific transfusion and anti-CD154 antibody. Transplantation 2004;77(9):1341-9.

13. Phelps CJ, Koike C, Vaught TD, Boone J, Wells KD, Chen SH, et al. Production of alpha 1,3-galactosyltransferase-deficient pigs. Science 2003;299(5605):411-4.

14. Biarnes M, Montolio M, Nacher V, Raurell M, Soler J, Montanya E. Beta-cell death and mass in syngeneically transplanted islets exposed to short- and long-term hyperglycemia. Diabetes 2002;51(1):66-72.

15. Pileggi A, Ricordi C, Alessiani M, Inverardi L. Factors influencing Islet of Langerhans graft function and monitoring. Clin Chim Acta 2001;310(1):3-16.

16. Davalli AM, Scaglia L, Zangen DH, Hollister J, Bonner-Weir S, Weir GC. Vulnerability of islets in the immediate posttransplantation period. Dynamic changes in structure and function. Diabetes 1996;45(9):1161-7.

17. Moritz W, Meier F, Stroka DM, Giuliani M, Kugelmeier P, Nett PC, et al. Apoptosis in hypoxic human pancreatic islets correlates with HIF-1alpha expression. Faseb J 2002;16(7):745-7.

18. Carlsson PO, Palm F, Andersson A, Liss P. Markedly decreased oxygen tension in transplanted rat pancreatic islets irrespective of the implantation site. Diabetes 2001;50(3):489-95.

19. Carlsson PO, Palm F, Mattsson G. Low revascularization of experimentally transplanted human pancreatic islets. J Clin Endocrinol Metab 2002;87(12):5418-23.

20. Efrat S, Fusco-DeMane D, Lemberg H, al Emran O, Wang X. Conditional transformation of a pancreatic beta-cell line derived from transgenic mice expressing a tetracycline-regulated oncogene. Proc Natl Acad Sci U S A 1995;92(8):3576-80.

21. Fleischer N, Chen C, Surana M, Leiser M, Rossetti L, Pralong W, et al. Functional analysis of a conditionally transformed pancreatic beta-cell line. Diabetes 1998;47(9):1419-25.

22. Lakey JR, Warnock GL, Shapiro AM, Korbutt GS, Ao Z, Kneteman NM, et al. Intraductal collagenase delivery into the human pancreas using syringe loading or controlled perfusion. Cell Transplant 1999;8(3):285-92.

23. Tsujimura T, Kuroda Y, Avila JG, Kin T, Oberholzer J, Shapiro AM, et al. Influence of pancreas preservation on human islet isolation outcomes: impact of the two-layer method. Transplantation 2004;78(1):96-100.

24. Wang T, Singh B, Warnock GL, Rajotte RV. Prevention of recurrence of IDDM in islet-transplanted diabetic NOD mice by adjuvant immunotherapy. Diabetes 1992;41(1):114-7.

25. Kilani RT, Mackova M, Davidge ST, Guilbert LJ. Effect of oxygen levels in villous trophoblast apoptosis. Placenta 2003;24(8-9):826-34.

26. Sladowski D, Steer SJ, Clothier RH, Balls M. An improved MTT assay. J Immunol Methods 1993;157(1-2):203-7.

27. Street CN LJ, Rajotte RV, Shapiro AMJ, Kieffer TJ, Lyon JG, Kin T, Korbutt GS. Enriched Human Pancreatic Ductal Cultures Obtained from Selective Death of Acinar Cells Express Pancreatic and Duodenal Homeobox Gene-1 Age-Dependently. The Review of Diabetic Studies 2004;1:66-79.

28. Emamaullee JA, Rajotte RV, Lakey JRT, Liston P, Korneluk RG, Shapiro AMJ, et al. XIAP Overexpression in Human Islets Prevents Early Post-Transplant Apoptosis and Reduces the Islet Mass Needed to Treat Diabetes. 2005;*Submitted.*

29. Emamaullee JA, Liston P, Korneluk RG, Shapiro AMJ, Elliott J. XIAP Overexpression in Islet Beta-cells Enhances Engraftment and Minimizes Hypoxia-Reperfusion Injury. Am J Transplant 2005;5(6):1297-305.

30. Trivedi N, Hollister-Lock J, Lopez-Avalos MD, O'Neil JJ, Keegan M, Bonner-Weir S, et al. Increase in beta-cell mass in transplanted porcine neonatal pancreatic cell clusters is due to proliferation of beta-cells and differentiation of duct cells. Endocrinology 2001;142(5):2115-22.

31. Liggins C, Orlicky DJ, Bloomquist LA, Gianani R. Developmentally regulated expression of Survivin in human pancreatic islets. Pediatr Dev Pathol 2003;6(5):392-7.

32. Gruslin A, Qiu Q, Tsang BK. X-linked inhibitor of apoptosis protein expression and the regulation of apoptosis during human placental development. Biol Reprod 2001;64(4):1264-72.

33. Straszewski-Chavez SL, Abrahams VM, Funai EF, Mor G. X-linked inhibitor of apoptosis (XIAP) confers human trophoblast cell resistance to Fas-mediated apoptosis. Mol Hum Reprod 2004;10(1):33-41.

34. Trudeau JD, Dutz JP, Arany E, Hill DJ, Fieldus WE, Finegood DT. Neonatal beta-cell apoptosis: a trigger for autoimmune diabetes? Diabetes 2000;49(1):1-7.

Chapter 6

Conclusions and Future Directions

6.1 Dissertation Findings and Implications

In the field of islet transplantation, it has been known for many years that a large proportion of the transplanted cells are lost to apoptosis during the early engraftment period. This phenomenon is seen even in the absence of an immune response, and it occurs as a direct result of oxidative stress, since post-transplant hypoxia is present continuously within the graft up until the time that revascularization is complete (1-8). Many studies targeted at enhancing islet survival during the early post-transplant period have been published, and a variety of different strategies have been tested. For example, overexpression of molecules known to enhance revascularization, such as vascular endothelial growth factor (VEGF), have been attempted, but these have yet to exhibit a significant impact on islet graft survival (9). This is likely related to the fact that although VEGF expression will hasten the revascularization process, it cannot provide an immediate benefit to the transplanted tissue. Other groups have tested several different molecules that inhibit the generation of and/or damage mediated by ROS, including GPx-1, SOD, and heme oxygenase-1 (HO). Although these molecules can protect islets during controlled in vitro challenges where ROS are specifically produced, the in vivo benefit of such an approach has been less clear (10-13). Likewise, anti-coagulation strategies aimed at inhibiting the IBMIR, using injection of activated protein C, or inhibition of thrombin, have shown only a modest benefit in vivo (14, 15). The failure of each of these approaches to significantly preserve islet mass post-transplant is most likely related to the fact that the islets are already quite stressed due to the isolation process. As such they are past the point of gaining overall benefit from molecules that block or ameliorate specific steps along the path to cell death, such as during the production of ROS, the IBMIR, or the later stages of chronic hypoxia.

Since the processes described above involve both extracellular (i.e. IBMIR) and intracellular (i.e. hypoxia and ROS) stimuli leading to β-cell apoptosis, another approach that has been taken is to directly inhibit the apoptotic cascade. A variety of apoptosis-associated proteins, including cFLIP, A20, BCL-2, and BCL-XL have proven to be effective in preventing apoptosis of islets in vitro following stimulation of specific cell death pathways (16-22). However, in terms of preventing islet apoptosis and graft loss in the early post-transplant period, results thus far have been largely disappointing. The molecules Bcl-2, Bcl-XL, and cFLIP have been shown to inhibit hypoxia and/or cytokine mediated β-cell death in vitro, but they confer at best a modest survival advantage in vivo (16-19, 22, 23). Only A20 has been shown to both protect β-cells from cytokine- and Fas-mediated killing in vitro and also to moderately reduce the islet mass required for syngeneic islet transplantation (20, 21). Taken collectively, these results suggest that the best strategy to enhance islet survival during the stresses of early post-transplant engraftment would be to block executioner caspases that function late in apoptosis, beyond the convergence point of both intracellular and extracellular signaling pathways.

For the studies presented in this thesis, the impact of overexpression of the caspase inhibitory protein XIAP during the early engraftment period was examined. Rationale for this approach was based on the fact that XIAP binds to the active site of all of the main effector caspases (caspases 3, 7, and 9), plus the fact that XIAP has proven to be effective in preventing apoptosis in other rodent models of disease, including ischemic injury to neurons (24-26). For reasons of experimental feasibility and ease, we chose to conduct our first investigations using a murine β-cell line, βTC-Tet. These cells grow very well in tissue culture (growth is Tet-regulatable) yet they retain a highly differentiated phenotype and the capacity to undergo glucose-stimulated insulin release. Given that XIAP must be expressed intracellularly to have its effect, we used a recombinant adenoviral vector to induce overexpression of the protein within βTC-Tet cells. Efficient gene delivery by this method resulted in expression of high levels of XIAP in virtually all cells. A key feature of this experiment was that βTC-Tet cells grow as a monolayer, and as such they are much easier to infect. They can also be infected to a much larger extent than β-cells within intact islets. Using this model system we determined that XIAP overexpression significantly enhanced β-cell survival in vitro during periods of stress due to hypoxia, hypoxia followed by reoxygenation, and cytokine insult. XIAP overexpression preserved both β-cell viability and glucose responsiveness, the latter being a critical function that ordinarily disappears very early on during hypoxic stress. These results were confirmed in vivo by the finding that the mean time to achieve normoglycemia in recipient mice was decreased nearly 7-fold when XIAP was overexpressed within β-cell grafts. This observation represents the first reported instance where delivery of an anti-apoptotic gene to βTC-Tet cells resulted in enhanced graft function in vivo. Our hypothesis that XIAP would be superior to other upstream anti-apoptotic genes in enhancing β-cell survival post-transplant was confirmed.

Based upon these positive results obtained using βTC-Tet cells, the impact of XIAP overexpression in human islets was investigated. We knew that the biggest hurdle in reproducing the results obtained with the β-cell line in intact islets would be in achieving efficient delivery of the XIAP gene, since an effect would only be observed when the majority of cells within each islet expressed the anti-apoptotic protein. One of the main reasons why adenoviral vectors were chosen for the studies described in this thesis is that these vectors have proven to be among the most reliable and efficient for delivering genes to intact islets. In contrast to lentiviral vectors, recombinant adenoviruses also have the advantage that they avoid non-targeted genomic integration, which could present a serious problem when delivering a known oncogene like XIAP. However, 'efficient' gene delivery to human islets is a relative term, since the best reported transduction efficiencies have been <50% (i.e. less than half of the cells transduced per islet) even when enormous quantities of virus were used (up to 500 V.P./cell, or approximately $5x10^5$ V.P./islet) (27, 28). Another limitation in these reports was the common observation of a 'shell effect', where the virus never penetrates to the core of the islet, so that cells within the most hypoxic and sensitive region of the islet remain untransduced. Also, at high viral loads, the vector itself becomes toxic to the islets, upregulating the expression of multiple chemokines and chemokine receptors, adding an additional negative variable to any in vivo studies (29, 30). Based on the Elliott lab's previous experience with mouse islets, we hypothesized that transduction of human islets very soon after isolation would enhance transduction efficiencies, allowing a lower quantity of virus to be used. Although unproven, this approach is based on the idea that soon after enzymatic digestion and isolation the capsule surrounding each islet remains disrupted, and this allows the recombinant viral particles to better penetrate into the islet substance through the capillary network before it collapses in vitro. After a series of pilot studies, we found that transduction of islets <1 h following the completion of the isolation procedure resulted in highly efficient gene transfer, with >95% of the islets showing some staining for X-GAL, and >75% of the cells within positive islets showing LACZ expression. This result was all the more notable considering that only 10 V.P./cell was used, whereas other groups reported using 50-100 fold more virus yet obtained at most 50% transduction efficiency in human islets (27-29).

Having achieved the transduction efficiencies described in the previous paragraph, it became feasible to test XIAP in human islets. Using the same in vitro system that we had used to induce hypoxia and hypoxia/reoxygenation in βTC-Tet cells, XIAP-transduced human islets were found to be significantly less apoptotic and to recover functional response to glucose stimulation following these insults. To challenge XIAP-tranduced human islets in vivo, a series of marginal mass islet graft transplants were performed in STZ-induced diabetic NOD-RAG$^{-/-}$ mice. We found that control grafts containing 2000 islets normalized blood glucose levels 100% of the time, while control grafts containing 1000 islets only restored euglycemia 10% of the time. When XIAP-transduced human islet grafts were used, 89% of animals became normoglycemic with only 600 islets, and these animals exhibited robust and stable serum human C-peptide levels that exceeded those observed in control animals that received 600 or even 1000 islets. Thus, inhibition of effector caspases via XIAP overexpression significantly enhanced human islet survival post-transplant, allowing a 70% reduction in the mass of human islets required to restore euglycemia.

Since NPI represent a potentially abundant and effective alternative tissue source for clinical islet transplantation, the benefit of XIAP overexpression in this tissue during the early engraftment phase was investigated. Surprisingly, pilot studies showed that control cohorts of NPI were completely resistant to hypoxia and reoxygenation in vitro, suggesting that adenovirally mediated XIAP overexpression would have nothing to add with respect to their survival capacity. Further systematic investigations showed that in vitro, NPI were naturally resistant to hypoxia and hypoxia-reoxygenation induced apoptosis, and that these stresses did not affect islet function. Also, very little evidence of apoptosis was found in NPI grafts at 24 h or 7 days post-transplantation into immunocompromised mice, demonstrating that unlike adult islets, NPI grafts are not lost to post-transplant apoptosis. This natural resistance could be attributed (at least in part) to high expression levels of endogenous XIAP, which disappears after the islet tissue matures in adult animals. This finding suggests that nature has utilized the potent effect of XIAP to protect neonatal islets, long before the studies presented in this thesis.

6.2 Using Caspase Inhibition to Promote Islet Survival in the Current Clinical Setting

6.2.1 Gene Delivery to Islets: Clinically Feasible?

The studies presented in this thesis suggest that NPI, a potential alternative islet source for transplantation, naturally express high levels of XIAP, removing the need to deliver and overexpress this potentially useful gene. However, the clinical implementation of NPI transplantation into humans remains controversial, and it may be some time before these controversies are resolved. According to the results of this thesis, ideally the currently used tissue source, cadaveric donor (and potentially living related donor) human islets should be modified to overexpress XIAP. In theory, an agent (e.g. small molecule) could be given that would somehow upregulate expression of the endogenous XIAP gene specifically within the islets, but the means by which to produce this effect is not evident. Thus, the best current option to enhance clinical islet survival using XIAP involves gene delivery. The primary obstacle here involves the transport of corrective genes both safely and efficiently to a majority of the islet cells, ideally with the end result being stable and regulatable gene expression. Many biophysical and biochemical methods such as electroporation, gene guns, liposomes, calcium phosphate, etc. have been tested on islets, but toxicity issues and low efficiencies, especially in intact islets, have lead to the abandonment of all of these strategies (reviewed in (31)). In the last few years, the focus on gene therapy vehicles in islet transplantation has revolved around viral vectors.

There are several properties that one must consider when choosing a virus for gene therapy to islets. First, the ideal viral vector would have a flexible cloning capacity, allowing for both smaller and larger gene inserts, and it would be able to infect post-mitotic cells. Second, it would be easily constructed and propagated to a high titre at a reasonable cost. Third, it would be able to maintain stable gene expression over long periods of time with no adverse immunological side effects. Although no known virus possesses all of these characteristics, adenovirus has been the most widely used vector to date since it does exhibit many of these properties.

Adenoviruses belong to a family of non-enveloped viruses with icosahedral capsid symmetry and a linear, dsDNA genome approximately 35-40 kb in length. Viral entry into host cells is mediated by an interaction between the Fiber (IV, or "spike") and a cellular receptor of unknown function called CAR, or 'coxsackievirus and adenovirus receptor' (32). After the virion enters the cell via endocytosis, it is transported to the nucleus. Once inside the nucleus, the early genes, including E1a and E1b, are expressed, leading to viral DNA replication and activation of the late genes, which encode capsid proteins (32). Viral assembly occurs in the nucleus, and accumulation of viral particles leads to host cell lysis (32). There are adenoviral gene products that have been shown to interact with the host cell environment to promote infectivity. E1b binds to and inactivates p53, an important protein involved in cell cycle regulation (32). E3 prevents cell surface expression of MHC class I by binding the peptide groove and sequestering the MHC in the endoplasmic reticulum, thus preventing detection of the virus by circulating cytotoxic T-lymphocytes (32). Finally, it should be noted that most immunological reactivity involves the capsid proteins, specifically Fiber (32).

In the late 1980's and early 1990's, a series of adenoviral vectors were produced that have since been termed 'first generation vectors'. In general, these were human adenovirus Serotypes 2 or 5, with the E1 and sometimes E3 regions of their genome deleted (33). These defects rendered them replication defective since the E1 proteins are required for activation of viral DNA replication. E3 was removed in an attempt to reduce immune interference. Since only a small portion of the genome had been removed, the insert size of foreign DNA was limited to a maximum of 8 kb. In order to grow the mutated viruses, they were packaged in 293 cells, which express the adenoviral E1 gene product in *trans*.

Strategies have also been developed that allowed for the complete removal of all of the adenoviral genome except for the packaging signals and the inverted terminal repeats, giving rise to the third generation vectors, also termed "gutless adenoviruses" or "helper dependent adenoviruses (HdAdV)" (33). Since these vectors have no viral genes, they possess a large cloning capacity of up to 38 kb, but also required a helper adenovirus encoding the viral genes in order to be grown in culture. Compared to the earlier vectors, the HdAdV pose virtually no risk of toxicity or immunogenicity other than that of the transgene. Also, with the introduction of the Cre-lox recombination system, the potential risk of helper virus contamination is less than 0.1%, which has markedly enhanced the safety of these vectors (34). Still, the technical challenges in preparing and propagating

HdAdV have made them less attractive, and most reports involving adenoviral vectors and islets have utilized first generation vectors.

As with many viruses, the outside of the virion contains the most antigenic proteins of adenovirus and mediates cellular entry; specifically, the Fiber, Hexon, and Penton capsid proteins have been shown to illicit the greatest immune response and interact with CAR to enter host cells (32). Due to varying levels of CAR expression in different tissues, altering the ligand-receptor interaction could not only increase the utility of the vector, but it could also provide a higher level of tissue specificity. Contreras et al. have constructed an adenoviral vector with modified tropism by the addition of RGD and pK7 motifs within the envelope fiber (29). This modification allows the virus to attach to a broader range of cells through integrins (RGD peptide) and cell-surface proteins containing polyanion motifs such as heparin sulfate (pK7 peptide, positively charged). Both of these receptor types are widely expressed in many different cell types, including β-cells (29). A βGal reporter virus (Ad-RGDpK7-LACZ) containing these modifications was shown to transduce >80% of human islets ex vivo with extremely low multiplicities of infection (≈0.1 V.P./cell). The reduced viral load resulted in lower toxicity and less inflammation following transplantation of the Ad-RGDpK7-LACZ transduced islets into chemically diabetic, immunodeficient NOD-SCID mice. It should also be noted that in the context of clinical immunosuppression (e.g. use of drugs like tacrolimus) the immunological reactivity to adenovirally transduced cells is prevented, reducing the risk of inflammation (35).

We believe that the method used in our studies to deliver XIAP to human islets could, at least in theory, be implemented directly in clinical islet transplantation. However, transduction of human islets is not a trivial task; in fact, we spent many months optimizing our method to obtain the positive data reported in this thesis. The transduction efficiency was dependent on the purity of the islet preparation, timing of adenovirus exposure post-isolation, and donor variability. Also, islets that have been transduced must be cultured for at least 24 h prior to transplantation in order to allow sufficient time for transgene expression. Despite our model to transduce the islets ex vivo, thereby reducing the risks of vector transfer to graft recipients, our data has clearly demonstrated that the possibility exists that some free virus could be present and could be transferred to the recipient. In our experiments with transduced islets, even with the relatively low MOI used, recombinant virus was still detectable up to 8 days post-transduction, and after more than twenty wash steps. This suggests that adenovirally transduced islets would have to be cultured for at least a week prior to transplantation to ensure that minimal free recombinant virus was transferred to the recipient. This free replication incompetent but 'infectious' virus presents a particularly troublesome situation when using XIAP, since its overexpression is associated with many cancers and when present it is generally a marker of a poor clinical prognosis (36). The fact that the patient would be considerably immunosuppressed could magnify this risk and compound the potential danger. There are a few strategies that could be employed to circumvent this problem. Several drug-regulated promoters have been indentified, including tamoxifen and tetracycline, which allow for easily regulated transgene expression in vivo (37). Thus, the patient could be given the drug only when the gene expression was needed, either during the first few weeks or months post-transplant or during a rejection episode, and the transgene would otherwise be turned off, removing its effect. Also, as mentioned previously, vectors could be designed with modified tropism that is islet-specific, although to date no such vector exists. Adding more controversy to the use of adenoviral vectors was a clinical trial at the University of Pennsylvania in 1999 which resulted in the untimely death of an otherwise healthy patient due to an acute, systemic immune response to the vector (38). For these reasons, alternative therapeutic strategies aimed at exerting the same effect of XIAP without the requirement for gene transfer would be a more attractive clinical option.

6.2.2 Alternative Strategies to Inhibit Effector Caspases

The in vivo benefit of XIAP overexpression compared to other anti-apoptotic molecules is related to its ability to potently block activation of multiple late, effector apoptotic molecules (i.e. caspases 3 and 7); this prevents β-cell death in the immediate post-transplant period, when multiple pro-apoptotic stimuli are present. However, application of XIAP clinically presents several major technical problems, most notably gene delivery and regulation as discussed above. In the context of improving β-cell engraftment, effector caspase inhibition may be required only transiently in the first few days or weeks following transplantation. A series of small molecule peptidyl protease inhibitors have been used for more than 15 years as tools to investigate the activity of caspases. One of the most potent of these is zVAD-FMK (N-benzyloxycabonyl-Val-Ala-Asp-fluoromethylketone; 'zVAD'), a pan caspase inhibitor that is cell-permeable and irreversibly binds to the active site of caspases 1-10 and 12 (Fig 6-1). Short course zVAD therapy has been used in several animal models of disease to prevent apoptosis of the affected tissues, including allergic airway inflammation, muscle ischemia-reperfusion injury, lupus nephritis, LPS-induced lung injury, amyotrophic lateral sclerosis, cerebral ischemia, and myocardial infarction (39-44). Despite its potential therapeutic impact, zVAD-FMK has not been taken into clinical trials due to fears that in the body FMK-modified peptides would be metabolized into fluoroacetate, a known toxin (45, 46). This effect has never been attributed to zVAD directly, and in vivo administration of zVAD at doses of 10 mg/kg/day for up to three weeks did not result in any reported adverse outcomes in mice (41, 47). The impact of zVAD therapy on islet survival in vitro (i.e. added to the tissue culture media) or following islet transplant (i.e. as a drug taken by the patient) remains unknown. Compared to XIAP, which requires 24-48 h for maximal expression, in vitro zVAD would be expected to exert an effect almost immediately, perhaps even during the isolation process, and in this regard it may be even more useful than XIAP.

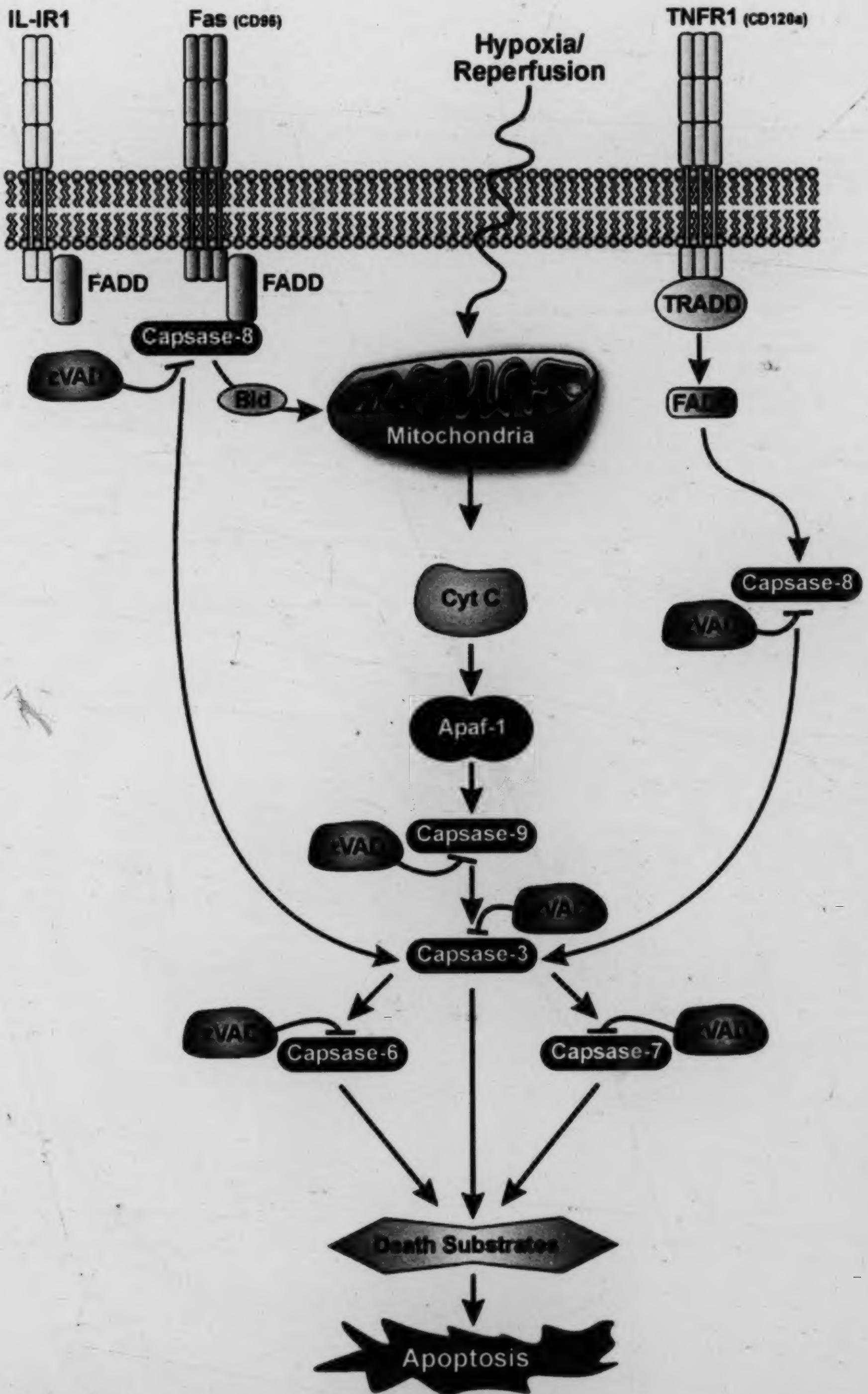


Figure 6-1: Pan Caspase Inhibitors (i.e. zVAD) can prevent activation of caspases, without the need for gene delivery.

6.3 Other Potential Benefits of Caspase Inhibitor Therapy in Islet Transplantation

Caspase inhibition via XIAP overexpression allows 70% fewer human islets to restore euglycemia in diabetic mice. This result implies that for untransduced islets, the majority of the transplanted tissue is dying following transfer to recipient animals, and the same phenomenon likely occurs in the established clinical model. In the context of allograft rejection, the overall 'state of health' or condition of the donor tissue has been implicated in directing the behavior of the immune response in the recipient. The 'danger' hypothesis suggests that death of donor tissue and its associated inflammatory response sends out warning signals to the immune system, resulting in activation of alloreactive T-cells (48-50). Conversely, when a graft is allowed to 'heal in' prior to immune reconstitution there are many fewer 'danger signals' being transmitted, so that by the time that the immune system does appear it is less activated and more likely to become tolerant to the allograft (48-50). Although this hypothesis has never been challenged in the face of an apoptosis-resistant graft (vs. a healed in graft), the model suggests that marked reduction in graft apoptosis should result in lower requirements for immunosuppression and a greater possibility for tolerance induction. Besides the indirect effect of preventing islet apoptosis on immunomodulation of allograft rejection, there also exists the possibility that exogenous caspase inhibitor therapy (i.e. zVAD) could directly inhibit the immune system (51).

In the context of each of the studies cited above, administration of zVAD to mice had no deleterious effect on normal cellular function in the lung, kidney, heart, liver, brain, or skeletal muscle, and it actually enhanced tissue survival in the face of stress, suggesting that caspase inhibitor therapies may not have any associated negative side effects. However, recent in vitro studies have shown that caspases play a critical role in cell cycle regulation of lymphocyte populations (51, 52). Initially, data obtained in FADD-deficient mice, which unexpectedly manifest a profound defect in T-cell proliferation, demonstrated that caspase activity itself may be required for lymphocyte proliferation (52). Studies conducted by Falk et al. using zVAD on human T-cells and peripheral blood mononuclear cells (PBMCs) confirmed this finding, since caspase inhibitors potently inhibited lymphocyte proliferation, recall antigen response, upregulation of MHC-II, CD25, and CD69, as well as IL-2 production following stimulation (51). Taken together, these findings suggest that caspase inhibition in vivo will reduce or prevent lymphocyte proliferation and activation. Although this has yet to be directly examined, results from studies using in vivo zVAD therapy targeted at preventing tissue death during inflammation have reported a marked decrease in infiltrating lymphocytes (40, 41, 53). While this result could be indirectly caused by reduced apoptotic death of the tissue (and therefore less infiltration), these findings nevertheless present the possibility that caspase inhibition may also prove to be a novel directly immunosuppressive agent in vivo, in addition to its potential benefits in terms of islet engraftment. In this regard, zVAD may work synergistically with known immunosuppressive agents, and therefore alter the therapeutic requirement for immunosuppression. If caspase inhibitor therapy could function to inhibit the immune system in vivo, it would be the first known immunomodulatory agent that also exerts a protective effect on the graft itself. Thus, further examination of the impact of effector caspase inhibition on established immunosuppressive and tolerance induction therapies is warranted.

6.4 Conclusions and Future Directions

In summary, the findings of this thesis strongly support the concept that inhibition of apoptosis at the level of effector caspases, using high levels of XIAP, promotes β-cell survival in islet transplantation. Adoption of this strategy in clinical islet transplantation could have an immediate impact at several levels. Reducing the islet mass required to achieve insulin independence would be a major clinical advance. Improvement in the reliability of single donor infusion, with the potential to treat two diabetic patients per donor pancreas, would broaden the availability of cadaveric islets. The latter accomplishment would also open up the possibility of living-related donors as a source of islet tissue. Also, single donor infusions would improve the safety of islet transplantation by reducing the risk of bleeding and portal vein thrombosis post-transplant, since less tissue would be infused during a single procedure. Enhancing islet engraftment post-transplant should prolong graft longevity, resulting in a more quiescent immunological state, thereby enhancing long-term rates of insulin-independence. Inhibition of islet apoptosis in the immediate post-transplant period may reduce the amount and intensity of anti-rejection therapy, accelerating 'accommodation' and drug minimization. If this could be achieved, or if stable immunological tolerance was enhanced through effector caspase inhibition therapy, either by XIAP overexpression or by exogenous caspase inhibitor administration, islet transplantation would be potentially safer and therefore more available to a broader spectrum of patients with T1DM, including children. Also, caspase inhibition may represent a novel strategy to suppress allorejection, with very few negative side effects outside the immune system, since caspases only appear to be involved in the cell cycle in lymphocyte lineage cells. Should caspase inhibitors function in vivo as directly immunosuppressive agents, this would herald the identification of a completely new therapy that both promotes graft survival, while also holding the immune system in check. In the broader sense, caspase inhibition therapies should hold promise for other types of cellular transplantation, including hepatocyte transplantation for metabolic disorders and neuronal cell transplantation for neurodegenerative disorders.

6.6 References

1. Biarnes M, Montolio M, Nacher V, Raurell M, Soler J, Montanya E. Beta-cell death and mass in syngeneically transplanted islets exposed to short- and long-term hyperglycemia. Diabetes 2002;51(1):66-72.

2. Dionne KE, Colton CK, Yarmush ML. Effect of hypoxia on insulin secretion by isolated rat and canine islets of Langerhans. Diabetes 1993;42(1):12-21.

3. Giuliani M, Moritz W, Bodmer E, Dindo D, Kugelmeier P, Lehmann R, et al. Central necrosis in isolated hypoxic human pancreatic islets: evidence for postisolation ischemia. Cell Transplant 2005;14(1):67-76.

4. Carlsson PO, Palm F, Andersson A, Liss P. Markedly decreased oxygen tension in transplanted rat pancreatic islets irrespective of the implantation site. Diabetes 2001;50(3):489-95.

5. Carlsson PO, Palm F, Mattsson G. Low revascularization of experimentally transplanted human pancreatic islets. J Clin Endocrinol Metab 2002;87(12):5418-23.

6. Davalli AM, Ogawa Y, Ricordi C, Scharp DW, Bonner-Weir S, Weir GC. A selective decrease in the beta cell mass of human islets transplanted into diabetic nude mice. Transplantation 1995;59(6):817-20.

7. Mendola JF, Goity C, Fernandez-Alvarez J, Saenz A, Benarroch G, Fernandez-Cruz L, et al. Immunocytochemical study of pancreatic islet revascularization in islet isograft. Effect of hyperglycemia of the recipient and of in vitro culture of islets. Transplantation 1994;57(5):725-30.

8. Stagner JI, Mokshagundam S, Samols E. Hormone secretion from transplanted islets is dependent upon changes in islet revascularization and islet architecture. Transplant Proc 1995;27(6):3251-4.

9. Narang AS, Cheng K, Henry J, Zhang C, Sabek O, Fraga D, et al. Vascular endothelial growth factor gene delivery for revascularization in transplanted human islets. Pharm Res 2004;21(1):15-25.

10. Moriscot C, Pattou F, Kerr-Conte J, Richard MJ, Lemarchand P, Benhamou PY. Contribution of adenoviral-mediated superoxide dismutase gene

transfer to the reduction in nitric oxide-induced cytotoxicity on human islets and INS-1 insulin-secreting cells. Diabetologia 2000;43(5):625-31.

11. Tobiasch E, Gunther L, Bach FH. Heme oxygenase-1 protects pancreatic beta cells from apoptosis caused by various stimuli. J Investig Med 2001;49(6):566-71.

12. Pileggi A, Molano RD, Berney T, Cattan P, Vizzardelli C, Oliver R, et al. Heme oxygenase-1 induction in islet cells results in protection from apoptosis and improved in vivo function after transplantation. Diabetes 2001;50(9):1983-91.

13. Lepore DA, Shinkel TA, Fisicaro N, Mysore TB, Johnson LE, d'Apice AJ, et al. Enhanced expression of glutathione peroxidase protects islet beta cells from hypoxia-reoxygenation. Xenotransplantation 2004;11(1):53-9.

14. Ozmen L, Ekdahl KN, Elgue G, Larsson R, Korsgren O, Nilsson B. Inhibition of thrombin abrogates the instant blood-mediated inflammatory reaction triggered by isolated human islets: possible application of the thrombin inhibitor melagatran in clinical islet transplantation. Diabetes 2002;51(6):1779-84.

15. Contreras JL, Eckstein C, Smyth CA, Bilbao G, Vilatoba M, Ringland SE, et al. Activated protein C preserves functional islet mass after intraportal transplantation: a novel link between endothelial cell activation, thrombosis, inflammation, and islet cell death. Diabetes 2004;53(11):2804-14.

16. Cottet S, Dupraz P, Hamburger F, Dolci W, Jaquet M, Thorens B. SOCS-1 protein prevents Janus Kinase/STAT-dependent inhibition of beta cell insulin gene transcription and secretion in response to interferon-gamma. J Biol Chem 2001;276(28):25862-70.

17. Cottet S, Dupraz P, Hamburger F, Dolci W, Jaquet M, Thorens B. cFLIP protein prevents tumor necrosis factor-alpha-mediated induction of caspase-8-dependent apoptosis in insulin-secreting betaTc-Tet cells. Diabetes 2002;51(6):1805-14.

18. Dupraz P, Rinsch C, Pralong WF, Rolland E, Zufferey R, Trono D, et al. Lentivirus-mediated Bcl-2 expression in betaTC-tet cells improves resistance to hypoxia and cytokine-induced apoptosis while preserving in vitro and in vivo control of insulin secretion. Gene Ther 1999;6(6):1160-9.

19. Dupraz P, Cottet S, Hamburger F, Dolci W, Felley-Bosco E, Thorens B. Dominant negative MyD88 proteins inhibit interleukin-1beta /interferon-gamma - mediated induction of nuclear factor kappa B-dependent nitrite production and apoptosis in beta cells. J Biol Chem 2000;275(48):37672-8.

20. Grey ST, Arvelo MB, Hasenkamp W, Bach FH, Ferran C. A20 inhibits cytokine-induced apoptosis and nuclear factor kappaB-dependent gene activation in islets. J Exp Med 1999;190(8):1135-46.

21. Grey ST, Longo C, Shukri T, Patel VI, Csizmadia E, Daniel S, et al. Genetic engineering of a suboptimal islet graft with A20 preserves beta cell mass and function. J Immunol 2003;170(12):6250-6.

22. Klein D, Ribeiro MM, Mendoza V, Jayaraman S, Kenyon NS, Pileggi A, et al. Delivery of Bcl-XL or its BH4 domain by protein transduction inhibits apoptosis in human islets. Biochem Biophys Res Commun 2004;323(2):473-8.

23. Cattan P, Rottembourg D, Cottet S, Tardivel I, Dupraz P, Thorens B, et al. Destruction of conditional insulinoma cell lines in NOD mice: a role for autoimmunity. Diabetologia 2003;46(4):504-10.

24. Liston P, Roy N, Tamai K, Lefebvre C, Baird S, Cherton-Horvat G, et al. Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature 1996;379(6563):349-53.

25. Petrin D, Baker A, Coupland SG, Liston P, Narang M, Damji K, et al. Structural and functional protection of photoreceptors from MNU-induced retinal degeneration by the X-linked inhibitor of apoptosis. Invest Ophthalmol Vis Sci 2003;44(6):2757-63.

26. Trapp T, Korhonen L, Besselmann M, Martinez R, Mercer EA, Lindholm D. Transgenic mice overexpressing XIAP in neurons show better outcome after transient cerebral ischemia. Mol Cell Neurosci 2003;23(2):302-13.

27. Deng S, Vatamaniuk M, Lian MM, Doliba N, Wang J, Bell E, et al. Insulin gene transfer enhances the function of human islet grafts. Diabetologia 2003;46(3):386-93.

28. Fenjves ES, Ochoa MS, Gay-Rabinstein C, Molano RD, Pileggi A, Mendez AJ, et al. Adenoviral gene transfer of erythropoietin confers cytoprotection to isolated pancreatic islets. Transplantation 2004;77(1):13-8.

29. Contreras JL, Wu H, Smyth CA, Eckstein CP, Young CJ, Seki T, et al. Double genetic modification of adenovirus fiber with RGD polylysine motifs significantly enhances gene transfer to isolated human pancreatic islets. Transplantation 2003;76(1):252-61.

30. Zhang N, Schroppel B, Chen D, Fu S, Hudkins KL, Zhang H, et al. Adenovirus transduction induces expression of multiple chemokines and chemokine receptors in murine beta cells and pancreatic islets. Am J Transplant 2003;3(10):1230-41.

31. Giannoukakis N, Robbins PD. Gene and cell therapies for diabetes mellitus: strategies and clinical potential. BioDrugs 2002;16(3):149-73.

32. Shenk T. Adenoviridae: the viruses and their replication. In: Knipe DM, Howley PM, editors. Field's Virology. Philadelphia: Lippinscott Williams and Wilkins; 2001. p. 2266-2300.

33. Parks RJ. Improvements in adenoviral vector technology: overcoming barriers for gene therapy. Clin Genet 2000;58(1):1-11.

34. Parks RJ, Chen L, Anton M, Sankar U, Rudnicki MA, Graham FL. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. Proc Natl Acad Sci U S A 1996;93(24):13565-70.

35. Ritter T, Lehmann M, Volk HD. Improvements in gene therapy: averting the immune response to adenoviral vectors. BioDrugs 2002;16(1):3-10.

36. Fong WG, Liston P, Rajcan-Separovic E, St Jean M, Craig C, Korneluk RG. Expression and genetic analysis of XIAP-associated factor 1 (XAF1) in cancer cell lines. Genomics 2000;70(1):113-22.

37. Xu ZL, Mizuguchi H, Mayumi T, Hayakawa T. Regulated gene expression from adenovirus vectors: a systematic comparison of various inducible systems. Gene 2003;309(2):145-51.

38. Ferber D. Gene therapy. Safer and virus-free? Science 2001;294(5547):1638-42.

39. Iwata A, Harlan JM, Vedder NB, Winn RK. The caspase inhibitor z-VAD is more effective than CD18 adhesion blockade in reducing muscle ischemia-reperfusion injury: implication for clinical trials. Blood 2002;100(6):2077-80.

40. Iwata A, Nishio K, Winn RK, Chi EY, Henderson WR, Jr., Harlan JM. A broad-spectrum caspase inhibitor attenuates allergic airway inflammation in murine asthma model. J Immunol 2003;170(6):3386-91.

41. Seery JP, Cattell V, Watt FM. Cutting edge: amelioration of kidney disease in a transgenic mouse model of lupus nephritis by administration of the caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-(beta-o-methyl)-fluoromethylketone. J Immunol 2001;167(5):2452-5.

42. Kawasaki M, Kuwano K, Hagimoto N, Matsuba T, Kunitake R, Tanaka T, et al. Protection from lethal apoptosis in lipopolysaccharide-induced acute lung injury in mice by a caspase inhibitor. Am J Pathol 2000;157(2):597-603.

43. Li M, Ona VO, Guegan C, Chen M, Jackson-Lewis V, Andrews LJ, et al. Functional role of caspase-1 and caspase-3 in an ALS transgenic mouse model. Science 2000;288(5464):335-9.

44. Huang JQ, Radinovic S, Rezaiefar P, Black SC. In vivo myocardial infarct size reduction by a caspase inhibitor administered after the onset of ischemia. Eur J Pharmacol 2000;402(1-2):139-42.

45. Eichhold TH, Hookfin EB, Taiwo YO, De B, Wehmeyer KR. Isolation and quantification of fluoroacetate in rat tissues, following dosing of Z-Phe-Ala-CH2-F, a peptidyl fluoromethyl ketone protease inhibitor. J Pharm Biomed Anal 1997;16(3):459-67.

46. Van Noorden CJ. The history of Z-VAD-FMK, a tool for understanding the significance of caspase inhibition. Acta Histochem 2001;103(3):241-51.

47. Clark P, Dziarmaga A, Eccles M, Goodyer P. Rescue of defective branching nephrogenesis in renal-coloboma syndrome by the caspase inhibitor, Z-VAD-fmk. J Am Soc Nephrol 2004;15(2):299-305.

48. Anderson CC, Carroll JM, Gallucci S, Ridge JP, Cheever AW, Matzinger P. Testing time-, ignorance-, and danger-based models of tolerance. J Immunol 2001;166(6):3663-71.

49. Matzinger P. Tolerance, danger, and the extended family. Annu Rev Immunol 1994;12:991-1045.

50. Matzinger P. An innate sense of danger. Semin Immunol 1998;10(5):399-415.

51. Falk M, Ussat S, Reiling N, Wesch D, Kabelitz D, Adam-Klages S. Caspase inhibition blocks human T cell proliferation by suppressing appropriate regulation of IL-2, CD25, and cell cycle-associated proteins. J Immunol 2004;173(8):5077-85.

52. Kennedy NJ, Kataoka T, Tschopp J, Budd RC. Caspase activation is required for T cell proliferation. J Exp Med 1999;190(12):1891-6.

53. Braun JS, Novak R, Herzog KH, Bodner SM, Cleveland JL, Tuomanen EI. Neuroprotection by a caspase inhibitor in acute bacterial meningitis. Nat Med 1999;5(3):298-302.

Appendix 1

Supplemental Data

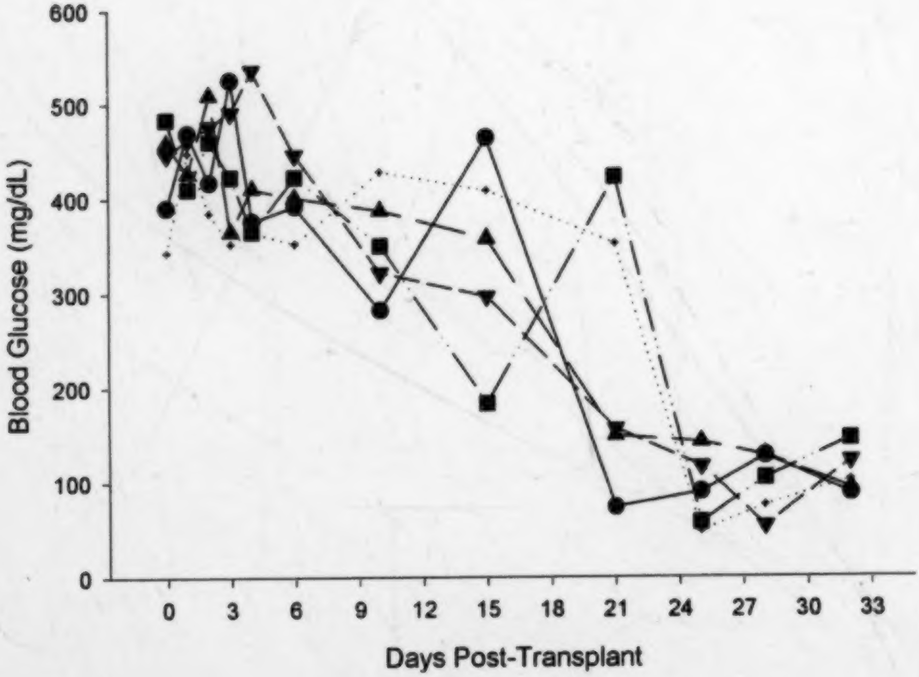


Figure A1-1: Untransduced βTC-Tet cells reverses diabetes in approximately 21 days following transplantation subcutaneously into chemically-diabetic NOD-RAG$^{-/-}$ mice.

Diabetes was induced in NOD-RAG$^{-/-}$ mice by streptozotocin injection. Six days later diabetic animals (blood glucose≥325 mg/dL) were transplanted with 4×10^6 untransduced βTC-Tet cells (N=54). The day of normoglycemia was defined as the first day where blood glucose was ≤220 mg/dL, provided that it remained ≤220 mg/dL on the next consecutive measurement. Animals that reached normoglycemia were maintained on doxycycline in order to arrest the growth of the transplanted βTC-Tet cells.

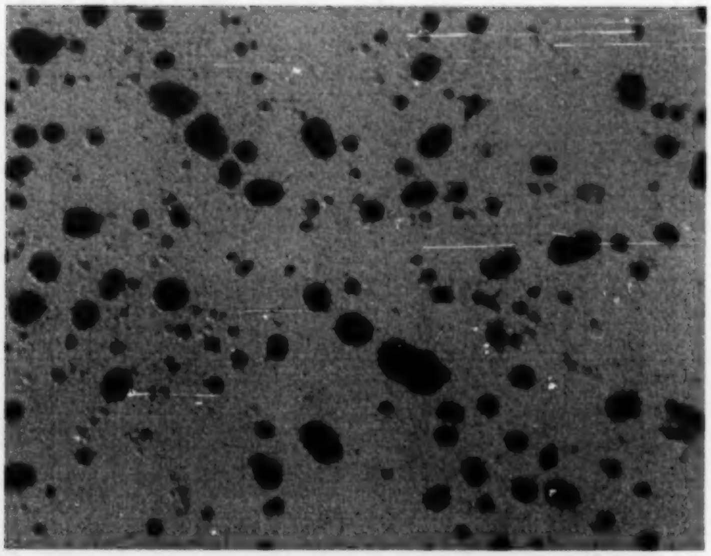


Figure A1-2: Representative preparation of transduced human islets that had been Dithizone-stained after 10 days in culture.

This photograph was taken after 30 min. of dithizone exposure. Human islets were transduced with either Ad-XIAP or Ad-LACZ at 10 m.o.i./cell (roughly 10^4/islet) <1 h. following isolation. After 24 h., the islets were washed twice with PBS and resuspended in fresh culture medium. The islets were washed every 48 h thereafter, until being transplanted at 10 days post-transduction.

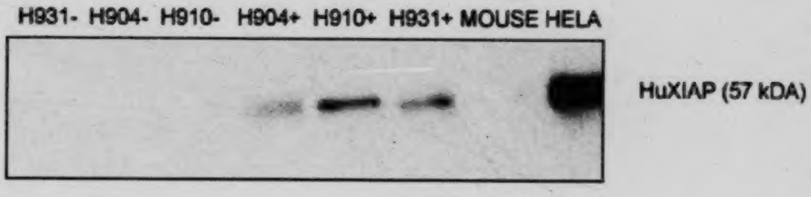


Figure A1-3: Untransduced human islets from preparations H904 and H931 also show no detectable HuXIAP.

(A) 10 μg of total protein from human islet lysates prepared from untransduced or Ad-XIAP transduced (H904, H910, H931) human islets (three independent preparations) were run alongside lysates made from two control cell lines. As a negative control, untransduced murine βTC-Tet cells (MOUSE) were used, since they do not produce any detectable Hu-XIAP. As a positive control, the lane labeled 'HELA' contains 10 μg protein lysate from Hela cells, which are known to express high levels of endogenous Hu-XIAP.

Figure A1-4: Human islet sections treated with PE-conjugated secondary antibody in the absence of anti-insulin primary antibody exhibited no detectable fluorescence.

As a negative control sections of embedded human islets were treated in parallel with PBS as those stained with anti-insulin. To detect bound anti-insulin antibody, both sets of slides were incubated with PE-conjugated anti-guinea pig IgG. To demonstrate that no PE-conjugated antibody had bound nonspecifically to the islet sections, islet sections were counterstained with the nuclear dye DAPI (blue), and examined using fluorescent microscopy. A representative islet section is shown, with no detectable red fluorescence.

Appendix 2

Creation and Characterization of Transgenic NOD Mice with β-cell Specific XIAP Overexpression

A2.1 Introduction

T1DM is a chronic progressive T-cell mediated autoimmune disease, which results when the immune system destroys the insulin-producing pancreatic β-cells within the islets of Langerhans. The β-cells are especially vulnerable to oxidative stress, and they are also easily killed when exposed to pro-inflammatory cytokines in vitro or by administration of free-radical generating agents such as streptozotocin or alloxan in vivo. Islet transplantation can be used to treat T1DM, but the islets are susceptible to apoptosis both during the stress of islet isolation and following transplantation (the latter being induced by both allo- and auto-immunity). Genetically modified islets that are resistant to rejection in the absence of immunosuppressive drugs would greatly improve the efficacy and availability of islet transplantation to patients with T1DM.

The NOD mouse provides a model of human autoimmune diabetes, and in these animals the β-cells are killed by $CD4^+$ and/or $CD8^+$ T cells primarily via a FAS-mediated apoptotic mechanism (1-3). In an effort to prevent autoimmune destruction of the islets in NOD mice, several different transgenic strategies have been investigated. These include β-cell specific overexpression of FasL, transforming growth factor-β, thioredoxin, TNFα, BCL-2, BCL-XL, adenoviral E3 proteins, and the viral anti-apoptotic gene CrmA (4-11). In terms of proving a benefit in inhibiting apoptosis in islet β-cells prior to the onset of autoimmunity, results have been inconclusive. Overexpression of BCL-2 did not prevent autoimmune destruction of β-cells, and BCL-XL overexpression caused a defect in mitochondrial glucose sensitivity, leading to impaired insulin secretion (4, 11).

Overexpression of the anti-apoptotic poxvirus protein CrmA did not alter diabetes incidence in NOD mice, most likely because it can only inhibit initiator caspases (7). The only anti-apoptotic transgenic approach that has proven to reduce diabetes incidence in NOD mice involves overexpression of the adenoviral E3 genes, but the long term impact of the presence of foreign proteins in β-cells has not been investigated (5).

For these reasons, strategies targeted at inhibiting effector caspases in order to prevent apoptosis induced following both intracellular and extracellular signals warrant further examination. There are a number of endogenous intracellular proteins that can inhibit apoptosis, and one of the most potent of these is XIAP. XIAP prevents the activation of caspases 3, 7 and 9, effector caspases that function late in apoptosis beyond the convergence point of many apoptotic pathways. In several models of neurodegenerative disease, significantly improved outcomes have been obtained when the target neuronal tissue has been engineered to resist apoptosis by transgenic overexpression of XIAP, and the same should be possible for pancreatic β-cells under attack by the immune system (12-14).

A2.2 Materials and Methods

A2.2.1 Transgenic Constructs and Generation of Mice

Several different transgenic strategies were employed, and as a result several constructs were generated (Fig. A2-1): pRIP2-IRES-HA-XIAP, pRIP2-HA-XIAP ('BBX'), pRIP-tTA (previously described and generously provided by Dr.

R. Flavell, Yale University (15)), and pTRE-6-myc-XIAP (pTRE2 from Clontech was used as a backbone).

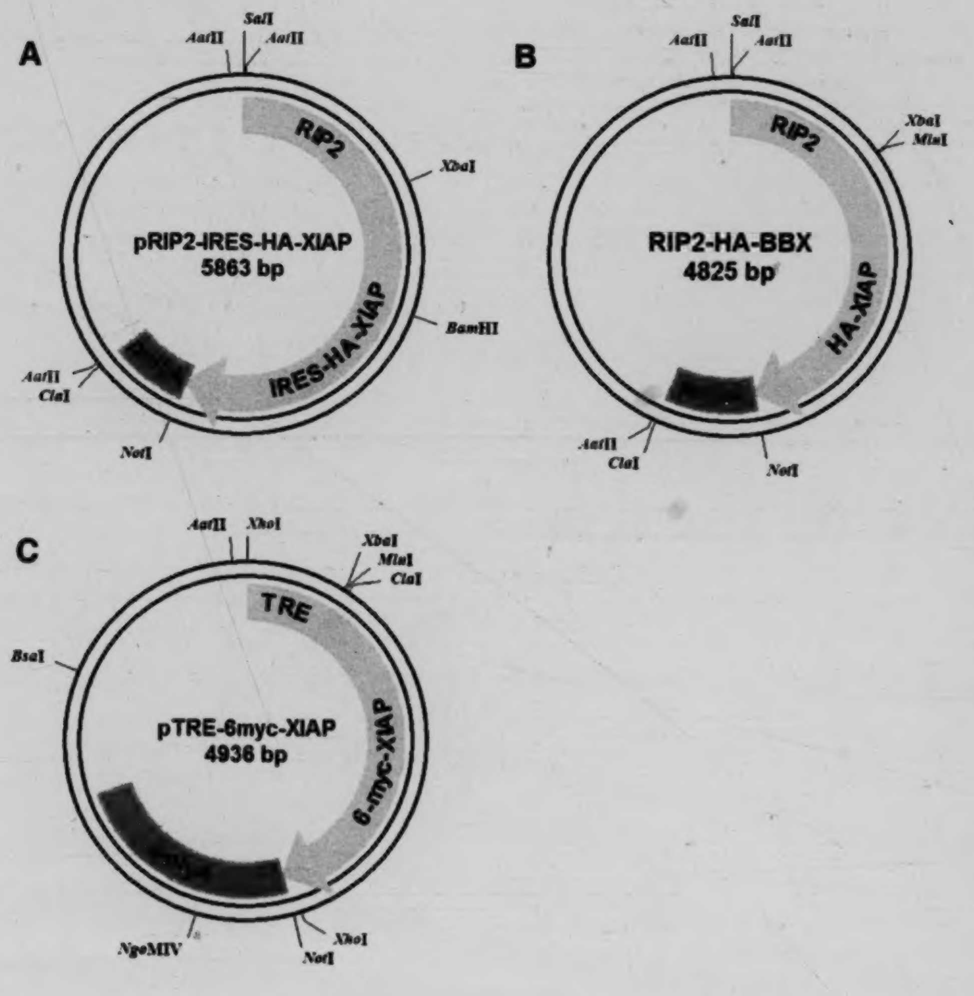


Figure A2-1: Plasmid constructs used to make transgenic NOD mice.

Plasmid maps--with relevant cloning sites and sites within cDNA inserts highlighted--of constructs used to generate transgenic NOD mice. pRIP-tTA is not shown, since it was provided without sequence information by Dr. R. Flavell and has been used successfully to generate tet-regulatable, β-cell specific transgene expression in NOD mice (15).

The first cohort of transgenic mice was created using the plasmid pRIP2-IRES-HA-XIAP, which encodes the recombinant human XIAP cDNA containing an IRES and HA (hemagluttinin) tag (obtained from P. Liston and R.G. Korneluk, University of Ottawa) driven by the rat insulin-II promoter (RIP2). For microinjection, the vector backbone was excised using Aat-II. The second series of transgenic mice were created using the plasmid pRIP2-HA-XIAP, which encodes the HA-tagged human XIAP cDNA driven by the rat insulin-II promoter (RIP2). The transgenic insert was excised by enzymatic digestion with Sal-I and Cla-I. The third series of transgenic mice were created using the Tet-inducible system, which requires two plasmid constructs. Plasmid pRIP-tTA encodes the tetracycline-responsive transactivator fused to the herpesvirus VP16 transcriptional transactivator under the control of the RIP promoter, and as such is only functional in the absence of tetracycline (or the more potent analog doxycycline). The transgenic insert was excised as described previously by enzymatic digestion with Not-I and Sca-I (15). Plasmid pTRE-6-myc-XIAP encodes the human XIAP cDNA with an N-terminal 6-myc epitope tag, driven by seven tetracycline-responsive operator sequences linked to the promoter sequences of the immediate-early gene of human cytomegalovirus. The transgenic insert from this plasmid was excised by enzymatic digestion with Aat-II, NgoM-IV, and Bsa-I.

Each transgenic construct was sequenced to confirm that no errors were incorporated during the cloning process. For all transgenic constructs, the transgenic plasmid fragment was separated from the plasmid backbone by agarose gel electrophoresis and purified from the resulting gel slice using the Qiaquick Gel Purification System (Qiagen). RIP2-IRES-HA-XIAP, RIP2- HA-XIAP, or a 1:1 ratio of both RIP-tTA and TRE-6-myc-XIAP transgenic inserts were microinjected into the pronuclei of NOD mice (performed by P. Dickie, transgenic core at the University of Alberta). Injected embryos were transferred to pseudopregnant FVB/N females to produce potential transgenic founders.

A2.2.2 Transfection of βTC-Tet Cells with Transgenic Constructs

Transient transfection of βTC-Tet cells was carried out using Lipofectamine 2000 (Invitrogen), according to the manufacturer's suggested protocol. Briefly, 1.5 μg plasmid DNA was combined with 4 μL Lipofectamine 2000, and complexes were allowed to form at room temperature for 20 min. These preformed complexes were added to a well containing 5×10^5 βTC-Tet cells in 12-well dishes. Following overnight incubation, the cells were washed, and at 48 h post-transfection, the cells were harvested for expression analysis. In general, approximately 50% transduction efficiency in βTC-Tet cells was observed when reporter constructs were used.

A2.2.3 Animals

NOD/Lt-J mice were obtained from Jackson Labs and housed under specific pathogen-free conditions. All mice were cared for according to the guidelines of the Canadian Council on Animal Care and ethical approval was obtained from the animal welfare committee at the University of Alberta. For Tet-off transgenic mice, all breeder females were maintained on a special diet containing 20 mg/kg Doxycycline ('Dox-Diet,' Bio-Serv International) to arrest XIAP transgene expression during embryonic development. After weaning, all transgenic mice were maintained on normal diet.

A2.2.4 PCR and Southern Blot Analysis for Genomic Integration of Transgenes

Genomic DNA was extracted from tail biopsies using the DNeasy kit (Qiagen). DNA samples were screened by polymerase chain reaction (PCR) using primers specific for regions upstream and downstream of the human XIAP cDNA (RIP 5': gtcgacgtccggactagtgatc; RIP 3': ttaggctgggggttactgaatc; Poly A 5': tttgtgaaggaaccttacttct; Poly A 3': acaaaccacaactagaatgcag) or for VP16 (to identify the RIP-ttA transgene; primers have been described (15)). The following cycle was used for all PCR analysis: 84°C for 1 min., 56°C for 1 min., 72°C for 2 min., repeated for a total of 30 cycles.

PCR results were confirmed and copy number determined by Southern blotting using a XIAP cDNA probe (Mlu-I/Not-1 fragment). Briefly, 3 μg genomic DNA was digested with Stu-1, and DNA fragments were separated by agarose gel electrophoresis. Following brief denaturation, the DNA was transferred to nitrocellulose. The 32-P labeled XIAP cDNA probe was hybridized to the blot using dextran sulfate at 37 C for 24 h, and after a series of washing steps, the blot was analyzed using a phosphorimager and film.

A2.2.5 Western Blot Analysis

Transgene expression was detected using the following antibodies: anti-XIAP mouse monoclonal antibody (Clone 48, BD Pharmingen), anti-c-myc mouse monoclonal antibody (clone 9E10, Sigma), and anti-VP16 rabbit polyclonal antibody (BD Clontech). Lysates were prepared from $5x10^5$ βTC-Tet cells (or 500 islets) in EDTA-free protease inhibitor cocktail (Roche) supplemented with 0.1% SDS, followed by BCA assays to determine protein concentration (Pierce). Western blot analysis was performed by running 15 μg total protein extract on a 10% SDS-PAGE gel, followed by transfer to nitrocellulose. The membranes were blocked for 1 h in PBS containing 0.05% Tween-20 (PBS-T) and 5% dry skim milk (blocking buffer), and then incubated for overnight with antibody diluted in blocking buffer. After washing the membranes in PBS-T, they were incubated with peroxidase-labeled goat anti-mouse IgG (XIAP or c-myc) or goat anti-rabbit IgG (VP16) (Amersham Pharmacia Biotech) at a 1:2500 dilution in blocking buffer for 1 h. The ECL Plus detection system (Amersham Pharmacia Biotech) was used with BioMax-MR film (Kodak) to visualize protein bands.

A2.2.6 Islet Isolation

Islets were isolated from 8-12 week old F1 animals in each transgenic line using collagenase digestion and Ficoll gradients (16).

A2.2.7 Quantitative RNA Analysis

Following islet isolation, samples were snap frozen in liquid nitrogen and shipped to our collaborators P. Liston and R. G. Korneluk at the University of Ottawa for quantitative RT-PCR analysis (Taqman). XIAP mRNA levels were measured using Hu-XIAP specific probes and compared to GAPDH mRNA levels in each sample.

A2.2.8 Diabetes Monitoring

Diabetes incidence was monitored in transgenic mice and compared to matched, non-transgenic littermate mice using a One-Touch glucometer (Johnson and Johnson). Animals were considered to be diabetic following two consecutive readings >18 mmol/L.

A2.3 Results

A2.3.1 Confirmation of Transgenic Construct Function by Transient Transfection into βTC-Tet cells.

To confirm the function of the RIP2 promoter and the recombinant DNA constructs, each plasmid was transiently transfected into βTC-Tet cells. This cell line was utilized since the RIP2 promoter is only functional in β-cells. Also, since this cell line was derived tetracycline-regulated transgenic mice, the pTRE-6myc-XIAP plasmid could be tested.

As shown in Figure A2-2, lysates prepared from untransfected βTC-Tet cells expressed no detectable XIAP protein ('βTC-'), while lysates prepared from βTC-Tet cells transfected with RIP2-IRES-HA-XIAP ('IRES-HA-XIAP'; the HA-tagged hu-XIAP band appears at 58 kDA), RIP2-HA-XIAP ('HA-XIAP') or TRE-6-myc-XIAP (6-myc-XIAP'; the 6-myc tagged hu-XIAP band appears at 67 kDa) expressed high levels of XIAP protein. As a positive control, lysates prepared from βTC-Tet cells transduced with Ad-XIAP were analyzed ('βTC+'); the hu-XIAP band appears at 57 kDa, since no epitope tag was present. These data confirmed that each of the transgenic constructs was functional.

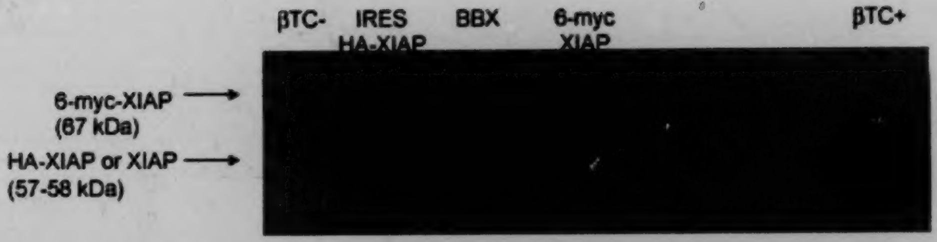


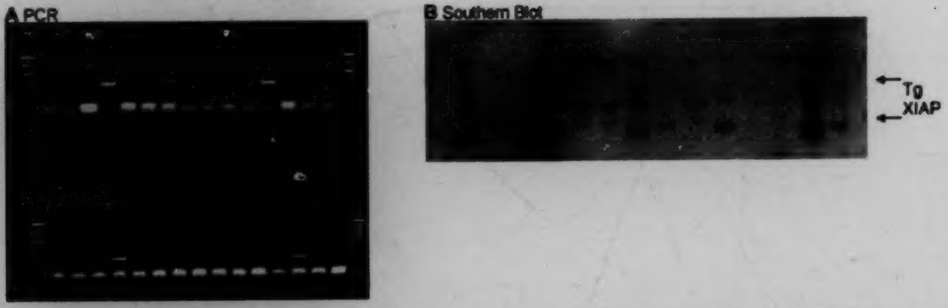
Figure A2-2: Transient transfection of transgenic plasmids into βTC-Tet cells demonstrated that each construct was functional.

Semiconfluent monolayers containing $5x10^5$ βTC-Tet cells were transfected with 1.5 μg plasmid using Lipfectamine 2000. 48 h following transfection, cells were harvested, and 15 μg protein lysate prepared from each sample was analyzed by SDS-PAGE and Western blotting for Hu-XIAP. Untransfected βTC-Tet cells (βTC-) were used as a negative control, since they do not produce any detectable Hu-XIAP. Lysates prepared from βTC-Tet cells transfected with RIP2-IRES-HA-XIAP ('IRES-HA-XIAP'; the HA-tagged hu-XIAP band appears at 58 kDA), RIP2-HA-XIAP ('HA-XIAP') or TRE-6-myc-XIAP (6-myc-XIAP'; the 6-myc tagged hu-XIAP band appears at 67 kDa) expressed high levels of XIAP protein. As a positive control, lysates prepared from βTC-Tet cells transduced with Ad-XIAP were analyzed ('βTC+'); the hu-XIAP band appears at 57 kDa, since no epitope tag was present. These data confirmed that each of the transgenic constructs was functional.

A2.3.2 Genomic Screening for Presence of Transgenes

To identify transgenic animals, tail biopsies were performed at 21-28 days of age, and genomic DNA was extracted. DNA samples were screened by PCR using primers specific for regions upstream and downstream of the human XIAP cDNA ('RIP' and 'PolyA' in Figure A2-3A) or for VP16 to identify the RIP-ttA transgene ('VP16' in Figure A2-3C). PCR results were confirmed and copy number determined by Southern blotting using a XIAP cDNA probe (representative blots shown in Figures A2-3B and A2-3D).

RIP-IRES-HA-XIAP or RIP-HA-XIAP Transgenic Mice



RIP-tTA/TRE-6-myc-XIAP Transgenic Mice

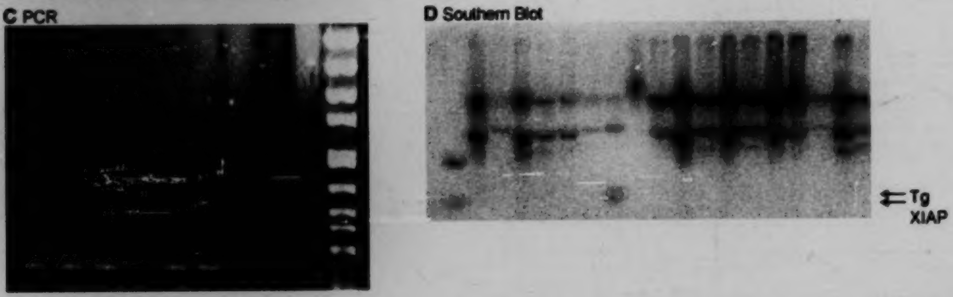


Figure A2-3: Representative PCR and southern blot analysis of construct integration in the genomic DNA of transgenic mice.

Tail biopsies were performed in 3-4 week old animals, and genomic DNA was extracted and purified. PCR analysis was carried out using primers upstream (A; 'RIP') and downstream (A; 'PolyA') of the XIAP cDNA, to confirm that the entire construct had integrated. To test for the presence of the tTA in tet-off mice, primers specific for VP16 were utilized (C). Representative Southern blots prepared from RIP-IRES-HA-XIAP (B) and RIP-tTA/TRE-6-myc-XIAP (D) transgenic mice are shown; the blots were analyzed with a XIAP cDNA probe. Transgenic bands are indicated with arrows.

In all, 7 unique founders were identified in the RIP2-IRES-HA XIAP line, and of these, four produced offspring (N1, N4, N7, N16). No RIP2-HA-XIAP founders were identified. For tet-off transgenic animals, three founders were identified: one was transgenic for the RIP-tTA contruct only (N13), one was transgenic for the

TRE-6-myc-XIAP construct only (N24; never produced offspring), and one was transgenic for both constructs (N7). Based upon results obtained with PCR and Southern blotting, animals were bred to generate animals homozygous for the transgene(s).

A2.3.3 Analysis of Islets in Transgenic Animals for XIAP mRNA or Protein Expression.

Initial founders were not examined for transgene expression so that they could be bred with wild-type NOD mice to generate F1 animals. Once F1 animals were generated, 5 animals from each line were randomly selected and sacrificed for islet isolation. For tet-off transgenic mice, animals were maintained without doxycycline for at least four weeks prior to expression analysis. Freshly isolated islets were either snap frozen and sent to measure Hu-XIAP mRNA levels by Taqman quantitative RT-PCR (performed by collaborators at the University of Ottawa), or protein lysates were prepared and analyzed using Western blotting. As shown in Figure A2-4, no difference in XIAP mRNA levels was observed between XIAP transgenic animals and non-transgenic NOD mice (representative result shown from animals in the RIP2-IRES-HA-XIAP N4 line; similar results were obtained in all founder lines analyzed).

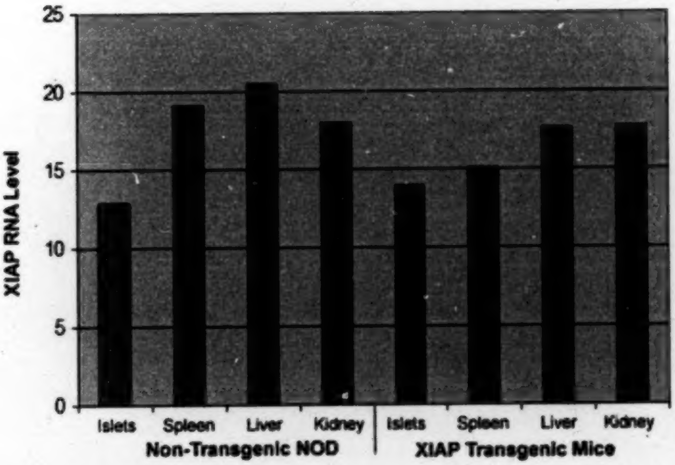
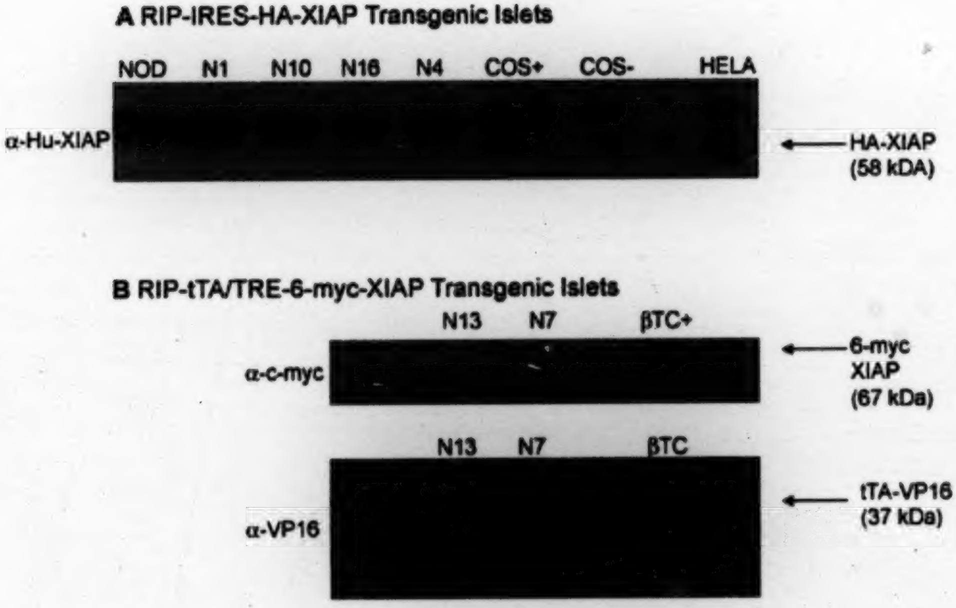


Figure A2-4: Representative quantitative RT-PCR for Hu-XIAP mRNA in islets isolated from transgenic mice.

Islets were isolated from F1 animals at 6-10 weeks of age, and frozen samples were sent to our collaborators in Ottawa for Taqman analysis using Hu-XIAP specific probes. GAPDH levels were also measured and used to standardize the samples. These results are representative of three independent experiments, and indicate that no Hu-XIAP mRNA could be detected, as compared to non-transgenic NOD mice.

These results were confirmed following repeat analysis of islet samples generated from three separate sets of F1 donors in each line. The negative results obtained by mRNA analysis were confirmed by Western blotting, where no detectable Hu-XIAP protein could be detected in any of the transgenic lines examined (Figure A2-5). Western blot analysis for the tTA-VP16 fusion protein demonstrated a faint band in line N7 (Figure A2-5B).



A RIP-IRES-HA-XIAP Transgenic Islets

B RIP-tTA/TRE-6-myc-XIAP Transgenic Islets

Figure A2-5: No Hu-XIAP protein could be detected in islets isolated from transgenic mice, although one line did exhibit low levels of the tTA protein.

Islets were isolated from F1 animals at 6-10 weeks of age, and 15 μg protein lysate prepared from each sample was analyzed by SDS-PAGE and Western blotting. (A) Lysates prepared from COS cells transfected with RIP2-IRES-HA-XIAP ('COS+; the HA-tagged hu-XIAP band appears at 58 kDA), were used as a positive control, and untransfected COS cells ('COS-') were used as a negative control, although a faint band does appear as a result of sample float-over from the COS+ lane. HELA cells, ('HELA') which naturally express high levels of Hu-XIAP, were also used as a positive control. None of the transgenic lines (N1, N4, N10, N16) produced detectable Hu-XIAP in their islets. A cross-reactive band which appears at approximately 60 kDa was shown as a loading control. (B) βTC-Tet cells transfected with TRE-6-myc-XIAP ('βTC+'; the 6-myc tagged hu-XIAP band appears at 67 kDa) were used as a positive control. Line N13 was used as a negative control, since it did not incorporate the TRE-6-myc XIAP construct. No detectable Hu-XIAP was present in line N7. To test for tTA expression in mice transgenic for RIP-tTA, anti-VP16 was utilized. As a positive control, lysates prepared from βTC-Tet cells ('βTC') were used, since this cell line originated in mice transgenic for tTA. The N7 line expressed detectable tTA protein. These blots are representative of three independent experiments.

A2.3.4 XIAP Transgenic Animals Exhibited No Difference in Prevalence of Diabetes, as Compared to Non-Transgenic NOD mice.

As predicted by the negative transgene expression data, none of the RIP2-IRES-HA-XIAP transgenic lines exhibited significant protection from diabetes onset, compared to nontransgenic NOD littermates (Figure A2-6). Diabetes incidence was not determined in tet-off transgenic mice, since it was observed early on that no detectable XIAP protein was being expressed in the islets.

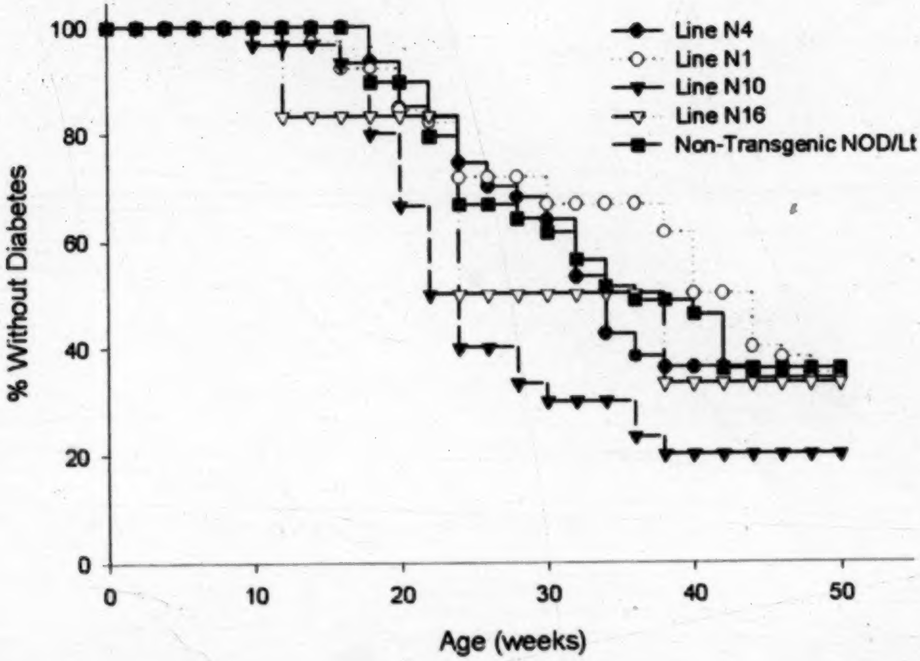


Figure A2-6: Diabetes incidence in XIAP transgenic mice did not differ from non-transgenic NOD mice.

F1 and F2 animals from each RIP2-IRES-HA-XIAP line (N1, N4, N10, N16) were followed for diabetes onset and compared to non-transgenic littermate NOD mice. No discernable difference between the lines and the non-transgenic mice (black squares) was observed.

A2.4 Discussion

Initially, three separate sets of injections were performed to generate potential RIP2-IRES-HA-XIAP founders (41 pups total). Tail DNA from each animal was subjected to PCR and Southern Blot analysis, and 7 independent founders were identified. Of these animals, 3 failed to produce offspring. The remaining four founders were bred to homozygosity, and the offspring were monitored for diabetes onset. On three occasions, islets from 8 week old animals in each line were isolated and examined for Hu-XIAP mRNA using Taqman RT-PCR and for XIAP protein using immunoblotting for both Hu-XIAP and the HA (haemagluttinin) tag. Unfortunately, even when high levels of HA-tagged XIAP could be detected following transient transfection into βTC-Tet cells, the HA epitope tag could never be detected by Western blot, despite utilization of numerous unique anti-HA antibodies. As such, no further experiments to detect the HA tag were carried out. The diabetes onset survival curves generated for each RIP2-IRES-HA-XIAP line showed no discernable difference when compared to non-transgenic NOD littermates (Fig. A2-6). These data are supported by the lack of XIAP expression as measured at both the RNA and protein level. Based on this data and discussions with our β-CAN colleagues (β-Cell Apoptosis Network; a JDRF-funded Canadian research group from 12/99-12/02)), we redesigned the XIAP construct by eliminating the IRES, which although is an endogenous element of XIAP, has been shown to interfere with XIAP expression in adenoviral mediated delivery as well as other transgenic models (e.g. neuron-specific; unpublished communication of P. Liston and R. G.

Korneluk). We termed this line "BBX," or "bare-bones XIAP," and although 30 potential founder pups were generated, none of the animals were transgenic.

The difficulties faced in generating viable transgenics that express XIAP led to the hypothesis that there might be low levels of insulin promoter activity during embryonic development, and in the case of XIAP any expression might interfere with development and be deleterious. We developed a new strategy using the Tet-inducible system that would allow for controlled XIAP expression. This approach had been proven to generate β-cell specific, regulated transgene expression in NOD-mice by other groups (15). We also chose to utilize a different epitope tag, a six repeat of c-myc, since our collaborators had experienced success in producing XIAP transgenic animals using this tag, which was easily detected and did not interfere with XIAP's function. In these animals, two separate constructs, RIP-tTA (expresses the TET-transactivator under the control of RIP), and TRE-6-myc-XIAP (expresses XIAP only in the presence of tTA and the absence of tetracycline) were co-injected, and 27 potential founders were generated. Of these pups, one was transgenic for both inserts (N7), one was transgenic for RIP-tTA (N13), and one was transgenic for TRE-6-myc-XIAP (N24). Only lines N7 and N13 produced offspring, and following a 4-week period off of doxycycline, expression analysis was carried out. Unfortunately, no detectable Hu-XIAP was present in line N7, and no detectable tTA was present in line N13 (Fig. A2-5). Further investigation into the N7 line, which had a male founder, revealed that only male mice in the F1 generation were transgenic. This suggested that the TRE-6myc-XIAP transgene had incorporated into the Y-chromosome, which is largely transcriptionally silent.

After these disappointing results were obtained, we decided to abandon the XIAP transgenic experiments (summarized in Table A2-1) and actively pursue islet transplantation experiments using Ad-XIAP instead. It should be noted that our colleagues in Ottawa attempted to generate RIP-HA-XIAP transgenic mice in the B6 background, and despite generating several founder lines, no Hu-XIAP protein was detected in the islets (P. Liston, unpublished communication). This supports our hypothesis that insulin-promoter regulated XIAP overexpression during development was selected against, and as a result only non-expressing founders could be generated. However, this hypothesis has yet to be directly investigated.

Table A2-1: Summary of Transgenic Lines Generated

Construct	**# of Injections; D.O.B.**	**# Potential Founders Generated**	**Founders with Offspring / Total Founders**	**Expression Analysis**	**Total Animals Screened**
RIP2-IRES-HA-XIAP	3 02/05/00 05/31/00 12/25/00	41	4/7	Western Blot (3x) Taqman (3x)	~470
RIP2-HA-XIAP	1 5/11/01	31	0/0	N.D.	31
RI[illegible]-tTA/ TRE-6-myc-XIAP	1 09/01/02	30	2/3	Western Blot (3x) Taqman (3x)	~100
TOTAL	**5**	**102**	**6/10**	**6 Isolations**	**~600**

Abbreviations used: D.O.B.- Date of Birth; N.D.- Not Determined.

A2.4 References

1. Suarez-Pinzon W, Sorensen O, Bleackley RC, Elliott JF, Rajotte RV, Rabinovitch A. Beta-cell destruction in NOD mice correlates with Fas (CD95) expression on beta-cells and proinflammatory cytokine expression in islets. Diabetes 1999;48(1):21-8.

2. Rabinovitch A, Suarez-Pinzon WL. Cytokines and their roles in pancreatic islet beta-cell destruction and insulin-dependent diabetes mellitus. Biochem Pharmacol 1998;55(8):1139-49.

3. Su X, Hu Q, Kristan JM, Costa C, Shen Y, Gero D, et al. Significant role for Fas in the pathogenesis of autoimmune diabetes. J Immunol 2000;164(5):2523-32.

4. Allison J, Thomas H, Beck D, Brady JL, Lew AM, Elefanty A, et al. Transgenic overexpression of human Bcl-2 in islet beta cells inhibits apoptosis but does not prevent autoimmune destruction. Int Immunol 2000;12(1):9-17.

5. Efrat S, Serreze D, Svetlanov A, Post CM, Johnson EA, Herold K, et al. Adenovirus early region 3(E3) immunomodulatory genes decrease the incidence of autoimmune diabetes in NOD mice. Diabetes 2001;50(5):980-4.

6. Grewal IS, Grewal KD, Wong FS, Wang H, Picarella DE, Janeway CA, Jr., et al. Expression of transgene encoded TGF-beta in islets prevents autoimmune diabetes in NOD mice by a local mechanism. J Autoimmun 2002;19(1-2):9-22.

7. Hugues S, Mougneau E, Ferlin W, Jeske D, Hofman P, Homann D, et al. Tolerance to islet antigens and prevention from diabetes induced by limited apoptosis of pancreatic beta cells. Immunity 2002;16(2):169-81.

8. Hotta M, Tashiro F, Ikegami H, Niwa H, Ogihara T, Yodoi J, et al. Pancreatic beta cell-specific expression of thioredoxin, an antioxidative and antiapoptotic protein, prevents autoimmune and streptozotocin-induced diabetes. J Exp Med 1998;188(8):1445-51.

9. Chervonsky AV, Wang Y, Wong FS, Visintin I, Flavell RA, Janeway CA, Jr., et al. The role of Fas in autoimmune diabetes. Cell 1997;89(1):17-24.

10. Savinov AY, Tcherepanov A, Green EA, Flavell RA, Chervonsky AV. Contribution of Fas to diabetes development. Proc Natl Acad Sci U S A 2003;100(2):628-32.

11. Zhou YP, Pena JC, Roe MW, Mittal A, Levisetti M, Baldwin AC, et al. Overexpression of Bcl-x(L) in beta-cells prevents cell death but impairs mitochondrial signal for insulin secretion. Am J Physiol Endocrinol Metab 2000;278(2):E340-51.

12. Trapp T, Korhonen L, Besselmann M, Martinez R, Mercer EA, Lindholm D. Transgenic mice overexpressing XIAP in neurons show better outcome after transient cerebral ischemia. Mol Cell Neurosci 2003;23(2):302-13.

13. Wang X, Zhu C, Hagberg H, Korhonen L, Sandberg M, Lindholm D, et al. X-linked inhibitor of apoptosis (XIAP) protein protects against caspase activation and tissue loss after neonatal hypoxia-ischemia. Neurobiol Dis 2004;16(1):179-89.

14. Crocker SJ, Liston P, Anisman H, Lee CJ, Smith PD, Earl N, et al. Attenuation of MPTP-induced neurotoxicity and behavioural impairment in NSE-XIAP transgenic mice. Neurobiol Dis 2003;12(2):150-61.

15. Green EA, Flavell RA. The temporal importance of TNFalpha expression in the development of diabetes. Immunity 2000;12(5):459-69.

16. Wang T, Singh B, Warnock GL, Rajotte RV. Prevention of recurrence of IDDM in islet-transplanted diabetic NOD mice by adjuvant immunotherapy. Diabetes 1992;41(1):114-7.